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**Nucleolin is involved in Epstein-Barr virus EBNA1-  
based mechanism of immune evasion and represents a  
relevant therapeutic target to treat cancers linked to  
this virus**

Maria José Lista

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**Nucleolin is involved in  
Epstein-Barr virus EBNA1-  
based mechanism of  
immune evasion and  
represents a relevant  
therapeutic target to treat  
cancers linked to this virus**

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# Abbreviations

## A

APC: antigen presenting cell

ARS: autonomous replicative sequences

Av: antigen variation

## B

BARTs: BamHI A rightward transcripts

BL: Burkitt lymphoma

## C

CD: complement receptor

CTL: cytotoxic T lymphocyte

## D

DBD: DNA binding domain

DFC: dense fibrillar compartment

DRiPs: defective ribosomal products

DS: dyad symmetry

## E

EBERs: Epstein-Barr-encoded small RNAs

EBNA: Epstein-Barr nuclear antigen

EBNA-LP: Epstein-Barr nuclear antigen leader protein

EBV: Epstein-Barr virus

ER: endoplasmic reticulum

## F

FC: fibrillar center

FR: family of repeats

## G

G4: G-quadruplex

GAr: glycine-alanine repeat

GAR: glycine-arginine repeat

GC: germinal center

GMP: genome maintenance protein

## H

HCMV: human cytomegalovirus  
HHV: human herpesvirus  
HIV: human immunodeficiency virus  
HL: Hodgkin lymphoma  
HLA: human leukocyte antigen  
HLC: classic hodgkin lymphoma  
hnRNP: heterogeneous nuclear ribonucleoprotein  
HPV: human papilloma virus  
HRS: Hodgkin-Reed Sternberg  
HSV: herpes simplex virus

## **I**

IF2 $\alpha$ : translation initiation factor 2 alpha  
IFN- $\gamma$ : interferon gamma  
Ig: immunoglobulin  
I $\kappa$ B: inhibitor of NF- $\kappa$ B  
IM: infectious mononucleosis  
IRES: internal ribosomal entry site

## **K**

KS: Kaposi sarcoma  
KSHV: Kaposi sarcoma-associated herpesvirus

## **L**

LCL: lymphoblastoid cell line  
LMP: latent membrane protein  
LP: lymphocyte predominant  
LTR: long terminal repeat

## **M**

MHC: major histocompatibility complex  
miRNA: microRNA  
MIIC: MHC class II loading compartment  
ML: multicentric Castleman's disease  
mRNA: messenger RNA

## **N**

NCL: nucleolin  
NHE: nuclease hypersensitive element  
NLPHL: nodular lymphocyte predominant HL  
NLS: nuclear localization signal  
NPC: nasopharyngeal carcinoma  
NSR1: nucleolin yeast homologue

## **O**

ORC: origin recognition complex  
OriP: origin of plasmid replication  
OVA: ovalbumin

## **P**

PEL: primary effusion lymphoma  
PRK: phosphoribulokinase

## **R**

RBD: RNA-binding domain  
rDNA: ribosomal DNA  
RPA: replication protein A  
rRNA: ribosomal RNA

## **S**

SHL8: SIINFKEL antigenic peptide derived from ovalbumin  
STAT1: signal transducer and activator of transcription 1

## **T**

TAP: transporter associated with antigen processing  
TCR: T-cell receptor  
TRAIL: TNF-related apoptosis inducing ligand  
TSS: transcription start site  
TPA: 12-*O*-tetradecanoylphorbol-13-acetate

## **U**

UTR: untranslated region

## **V**

VEGF: vascular endothelial growth factor  
VZV: varicella-zoster virus

## **Z**

ZRE: ZEBRA response element



# Abstract

The Epstein-Barr Virus (EBV) is the first oncogenic virus described in humans. It is a ubiquitous virus which infects over 90% of the human population.

EBV causes a latent infection of B lymphocytes and remains asymptomatic in most of infected individuals. However, in some conditions like immunosuppression, EBV can cause uncontrolled cell proliferation responsible for some types of cancers like Burkitt lymphoma, Hodgkin lymphoma and nasopharyngeal carcinoma.

During latency, EBNA1 is the only protein expressed in all EBV-infected cells as it is essential for EBV genome replication and maintenance. EBNA1 is highly antigenic and T cells raised against EBNA1 exist; nevertheless EBV-infected cells are not eliminated by the host immune system. This is due to the central GAR (Gly-Ala repeat) domain of EBNA1 which is able to inhibit the translation of its own mRNA *in cis*, thereby preventing the recognition of EBV-infected cells by the immune system of the host. The mechanisms involved in this GAR-based translation inhibition were unknown when I started my PhD.

During this thesis, we identified the first cellular factor, Nucleolin (NCL), able to affect GAR-based translation inhibition. We showed that NCL overexpression enhances EBNA1 translation inhibition and inversely, that its downregulation decreases EBNA1 translation inhibition effect. Furthermore, we demonstrated that the interaction between NCL and GAR RNA occurs through NCL binding to GAR-encoding mRNA *via* a particular secondary structure called G-quadruplex (G4). We showed that disrupting NCL-GAR RNA interaction with small G4 ligands enhances EBNA1 translation and thus antigen presentation, which constitutes a new therapeutic avenue to treat EBV-associated cancers. Finally, we went deeper into the mechanism involved in this interaction by showing that NCL has a direct effect on the GAR-based translation inhibition and that the nuclear location of NCL-GAR RNA interaction is forced by the nuclear location of NCL in the cells.

Taken together, these results have shed light upon the mechanism involved in the GAR domain translation inhibition and immune evasion, and have revealed a possible therapeutic target to eradicate EBV-associated cancers.



# Introduction

During my PhD, I studied the mechanism by which the Epstein-Barr virus is able to establish a lifelong infection of memory B cells without being detected by the host immune system. In line with this, I went deeper into the mechanism by which the central Gly-Ala (GAR) domain of EBNA1 protein inhibits the translation of its own mRNA *in cis* and, in this way escapes from T cell recognition and elimination by the host immune system. In the introduction section, I will first focus on EBV: its discovery, its life cycle, the diseases which are associated to its infection. In the second chapter, I will present EBV essential protein EBNA1: the central function of EBNA1 GAR domain will be deeply addressed as well as the possible mechanisms by which EBNA1 allows EBV infected cells to evade the immune system thanks to its GAR domain. In the third part of the introduction, I will present the yeast model that recapitulates the GAR-based inhibition of translation.

## CHAPTER I: The Epstein-Barr virus (EBV)

### 1. Discovery of the first human oncogenic virus: EBV

From the beginning of the 90's many observations showed evidence in favor of viruses related to human cancers. Nevertheless it was before 1957 that Denis Burkitt made its first observation in Africa of a strange form of lymphoma which was later called Burkitt lymphoma. This pathology was very common in young children in Africa and was fatal after metastasis. D. Burkitt was fascinated by this new type of lymphoma which led him to make a 10 weeks travel during which he determined a specific pattern of distribution of this disease in 12 countries. After analyzing all collected data, he realized that the distribution of this lymphoma corresponded to the location of malaria and yellow fever mosquito. He published his initial results in 1958 <sup>6</sup>. In 1961 he was invited to give a lecture in the Middlesex Hospital in London where he had the opportunity to meet Anthony Epstein. A. Epstein was fascinated by D. Burkitt's results and they started to collaborate. However it was only in 1963 when Yvonne Barr and Burt Achong joined A. Epstein's laboratory that they started to observe EBV's particles in biopsies taken from African patients <sup>7</sup>. Just after EBV's discovery, the Henles couple located in Philadelphia received some culture samples from A. Epstein and observed that antibodies against this new virus were present in children with Burkitt lymphoma but also in healthy children from Africa, demonstrating that EBV was widely distributed <sup>8</sup>. Nowadays we know that more than

90% of the human population is latently infected by EBV and that the infection remains asymptomatic in most infected people.

## 2. EBV belongs to the family of *herpesviridae*

Herpesviruses are DNA double-stranded viruses. Their genomes do not integrate into host DNA but persist in infected cells as extrachromosomal circular episomes. They are ubiquitous in the animal kingdom and they are the causing agents of lymphomas and immunodeficiency in humans. Eight members of this family have been identified in humans so far and all of them share a common origin as their genomes display high amino acid sequence similarity for many of their viral gene products<sup>9</sup>. Herpesviruses can be classified in three subfamilies: (1) **Alphaherpesviruses ( $\alpha$ )**, (2) **Betaherpesviruses ( $\beta$ )** and (3) **Gammaherpesviruses ( $\gamma$ )** depending on genome organization and sequence similarity.

These three families are presented below:

- (1) **The Alphaherpesviruses** include herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2) and the varicella-zoster virus (VZV). All of them are able to establish latent infection in sensory neurons, but can also infect several other types of human cells like mucosa and skin cells<sup>10</sup>.
- (2) **The Betaherpesviruses** include cytomegalovirus (HCMV or HHV-5) and three human roseolaviruses (HHV-6A, HHV-6B and HHV-7). They are able to latently infect myeloid cells.
- (3) **The Gammaherpesviruses**: In humans there are two  $\gamma$ -herpesviruses, the Epstein-Barr virus (EBV or HHV-4) which corresponds to the lymphocryptovirus genus and the Kaposi-sarcoma associated virus (KSHV or HHV-8) which corresponds to the rhadinovirus genus. Both of them are lymphotropic viruses that are able to latently infect B cells and epithelial cells. They establish a lifelong asymptomatic infection of their host with some intermittent lytic periods<sup>9</sup>. These two oncogenic viruses have been associated to some human cancers: EBV infection can cause Burkitt lymphoma (BL), Hodgkin lymphoma (HL), nasopharyngeal carcinoma (NPC) and some gastric cancers, whereas KSHV has been initially associated to Kaposi sarcoma (KS) and then also to Multicentric Castleman's disease (ML) and primary effusion lymphoma (PEL)<sup>11</sup>.

### 3. EBV-associated diseases

Most EBV infections are lifelong and asymptomatic; however many human diseases have been associated to EBV. In some cases EBV primary infection causes an acute infection described as infectious mononucleosis most commonly in teenagers and young adults. Moreover, several types of cancers have been associated with EBV, the most frequent being Burkitt and Hodgkin lymphomas and the epithelial nasopharyngeal carcinoma (**Table 1**).

**3.1 Infectious mononucleosis (IM):** IM was first described in 1920 by T.P. Sprunt and F.A. Evans as a new syndrome characterized by an acute infection with abnormal peripheral blood lymphocytes morphology, which were later shown to correspond to activated T CD8<sup>+</sup> cells responding against EBV infected cells <sup>12</sup>.

This disease is characterized by a global expansion of T CD8<sup>+</sup> cells raised against EBV-derived antigens. The most common symptoms are fever, fatigue, cervical lymph node enlargement and sore throat. IM is transmissible *via* saliva explaining why it is commonly called the “kissing disease”. Nevertheless, it can also be transmitted by blood transfusion, solid organ or hematopoietic cell transplantation.

**3.2 Burkitt lymphoma (BL):** It is the first cancer that has been linked to EBV infection. It was first described in 1958 as a lymphosarcoma present in young children in tropical Africa <sup>6</sup>. After EBV discovery, it was observed that most BL cases in equatorial Africa carry this virus <sup>13</sup>. Burkitt lymphomas are classified in three different groups: the endemic, the sporadic and the HIV-associated forms. More than 90% of endemic BL are EBV-positive while only 5-15% of BL are sporadic and 40% are associated to HIV infection. All BL are characterized by the chromosomal translocation of the c-Myc locus from its normal localization on chromosome 8 to one of the immunoglobulin (Ig) heavy or  $\kappa$  and  $\lambda$  light chain loci located on chromosomes 14, 2 or 22, respectively. Due to these translocations, the oncogene c-Myc finds itself under the transcriptional control of the Ig promoter causing its overexpression, hence leading to the high BL cells proliferation observed <sup>14</sup>. Most of BL present a latency I EBV profile, and some of them present a latency III profile (see section 4.2 below).

**3.3 Hodgkin's lymphoma (HL):** This type of lymphoma is more common in undeveloped countries like Peru or Kenya (57-59% of incidence) than in developed countries like North America where it represents between 20-50% of the total lymphomas <sup>15</sup>. HL are classified as classic HL (HLC) or nodular lymphocyte predominant HL (NLPHL) based mostly on the morphology and immunophenotype of the

malignant cells. HLC accounts for at least 95% of HL cases and is characterized by the presence of only 0,1-10% of malignant Hodgkin-Reed Sternberg (HRS) cells and the disruption of the lymph nodes<sup>16</sup>. NLPHL cases represent less than 5% of HL cases and are characterized by the presence of lymphocytes predominant cells (LP). HRS and LP cells present a deregulation of B cell specific genes that can be either very weakly expressed or not expressed at all. This is the case of key genes as B cell receptor BCR, CD19 and CD20. Both cell types also show a strong constitutive expression of NF- $\kappa$ B which contributes to the survival of cancer cells<sup>17</sup>. In general, HL shows a latency II profile of EBV infection (see on section 4.2 below).

**3.4 Nasopharyngeal carcinoma (NPC):** NPC is a rare epithelial head and neck cancer with an incidence in most part of the world of 1- 100.000 cases per year. Nevertheless, NPC is very common in Southern China and in Tunisia, where its prevalence is 25-30 cases per 100.000 people every year and represents one of the most predominant cancers among men<sup>18</sup>. All NPC are associated with EBV. It mostly occurs in males of about 40-60 years old. NPC is highly aggressive with an extensible local infiltration and early metastasis. There are three types of NPC: keratinizing squamous cell carcinoma, nonkeratinizing and basaloid carcinomas. The nonkeratinizing carcinoma is the most common one and is fully associated with EBV infection. It seems to be due to the proliferation of a single EBV-infected cell clone in the nasopharyngeal epithelium<sup>19</sup>. NPC carcinomas show a latency II profile of EBV infection (see on section 4.2 below).

**3.5 Gastric carcinomas:** between 2 and 20% of all gastric cancers are associated with EBV infection in epithelial cells with a world average of 10%. It is most common in males and the predominant localization is the proximal stomach. This type of cancer is associated to a latency II program (see on section 4.2 below). The most common factor associated to gastric cancers is infection by *Helicobacter pylori*. However, *H. pylori* infection is not a risk factor for gastric carcinoma associated to EBV which means that both pathogens are involved in different carcinogenic pathways<sup>20</sup>.

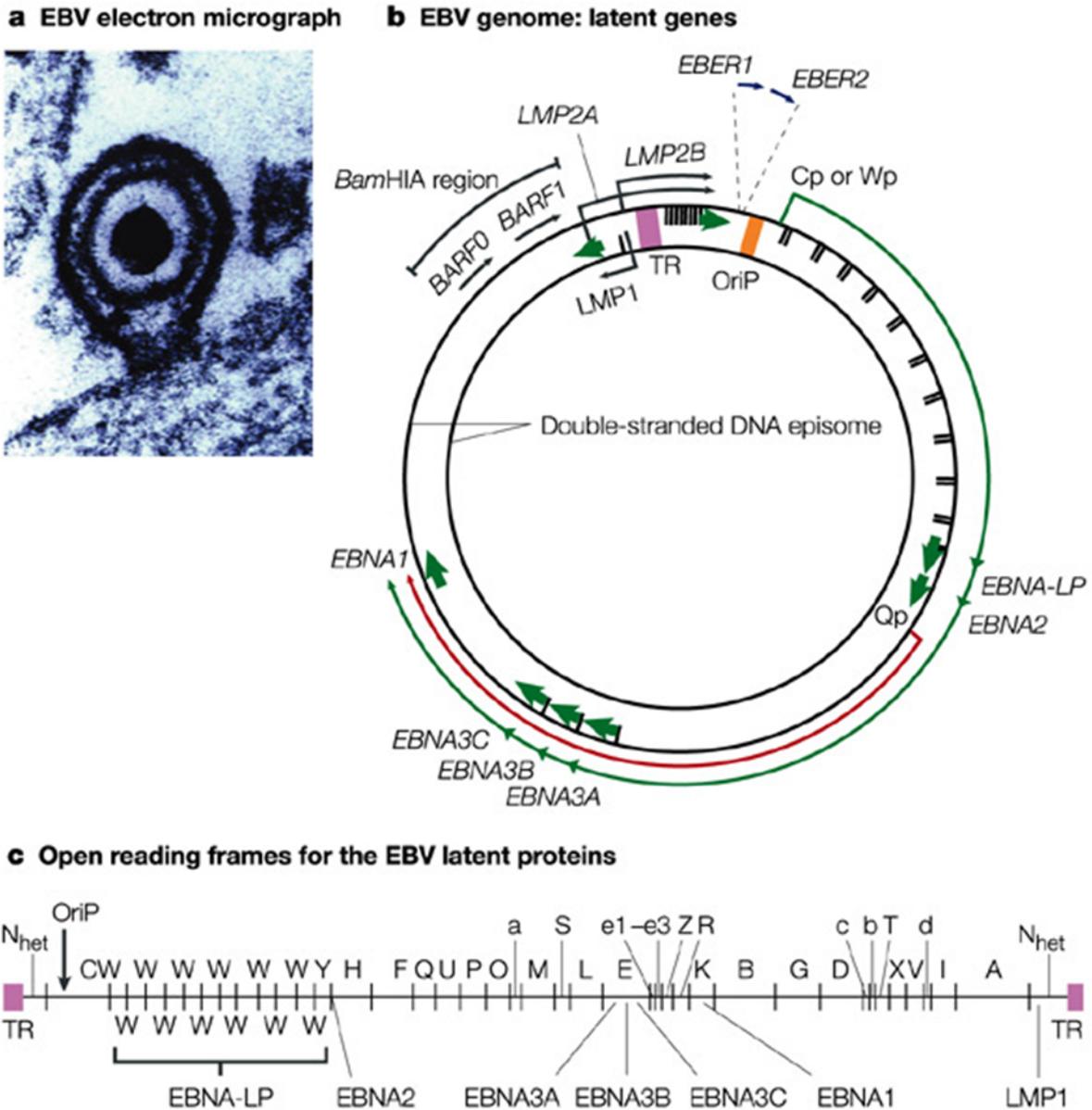
**3.6 NK/T-cell lymphomas:** they are rare aggressive non B cell tumors which are mostly common in Asia and Latin America. EBV expression pattern corresponds to a latency II program (see on section 4.2 below) with variable LMP1 expression, which contributes to the excessive production of pro-inflammatory cytokines mediated by NF- $\kappa$ B activation<sup>21</sup>.

**Table 1-** EBV-associated cancers. Table extracted from <sup>4</sup>.

| Disease   | Characteristics  |
|---|--|
| Burkitt's lymphoma  | <u>B cell origin</u><br>Endemic form in Africa is 100% EBV-associated<br>Only EBNA1 latent protein is expressed (latency I)                                      |
| Hodgkin's lymphoma  | <u>B cell origin</u><br>Common in western countries (USA and Europe)<br>40% EBV-associated<br>EBNA1, LMP1 and LMP2A/B latent proteins are expressed (latency II) |
| Lymphomas in immunosuppressed individuals (AIDS, post-transplant patients)  | <u>B cell or T cell origin</u><br>100% EBV-associated<br>EBNA1, EBNA2, EBNA3A/B/C, EBNA-LP, LMP1 and LMP2A/B latent proteins are expressed (latency III)         |
| Nasopharyngeal carcinoma  | <u>Epithelial cell origin</u><br>Common in south-east China<br>100% EBV-associated<br>EBNA1, LMP1 and LMP2A/B latent proteins are expressed (latency II)         |
| T/NK cell lymphomas (nasal T/NK lymphoma, aggressive NK-cell leukaemia, T cell lymphoproliferative disorder of childhood) | <u>T or NK cell origin</u><br>Common in Asian and Latin American countries<br>EBNA1, LMP1 and LMP2A/B latent proteins are expressed (latency II)                 |
| Gastric carcinoma   | <u>Epithelial cell origin</u><br>10% EBV-associated<br>EBNA1, LMP1 and LMP2A/B latent proteins are expressed (latency II)  |

#### 4. EBV life cycle

EBV was the first described human oncogenic virus and the first virus to have its genome totally cloned and sequenced. Its genome is composed of a double stranded DNA of approximately 180 kb which encodes approximately 80 proteins, from which only few have been characterized yet (**Figure 1b-c**). EBV genome is enclosed in an icosahedral nucleocapsid surrounded by a lipid envelope derived from the nuclear membrane (**Figure 1a**). EBV has the property to immortalize B cells in culture, and cell lines derived from this immortalization process are called lymphoblastoid cell lines (LCLs). As of today, EBV infection remains one of the most efficient mean to immortalize B cells *in vitro*.



**Figure 1.** Diagram showing an EBV virion and the localization of EBV latent genes on the viral DNA episome.

(a) Electron microscopy of the Epstein-Barr virus (EBV) virion, (b) EBV viral episome, (c) representation of EBV latent proteins open reading frames. The origin of replication (oriP) is shown in orange, the ORFs encoding each of the latent proteins are represented by violet arrows and the direction represents the transcription sense. Scheme extracted from <sup>22</sup>.

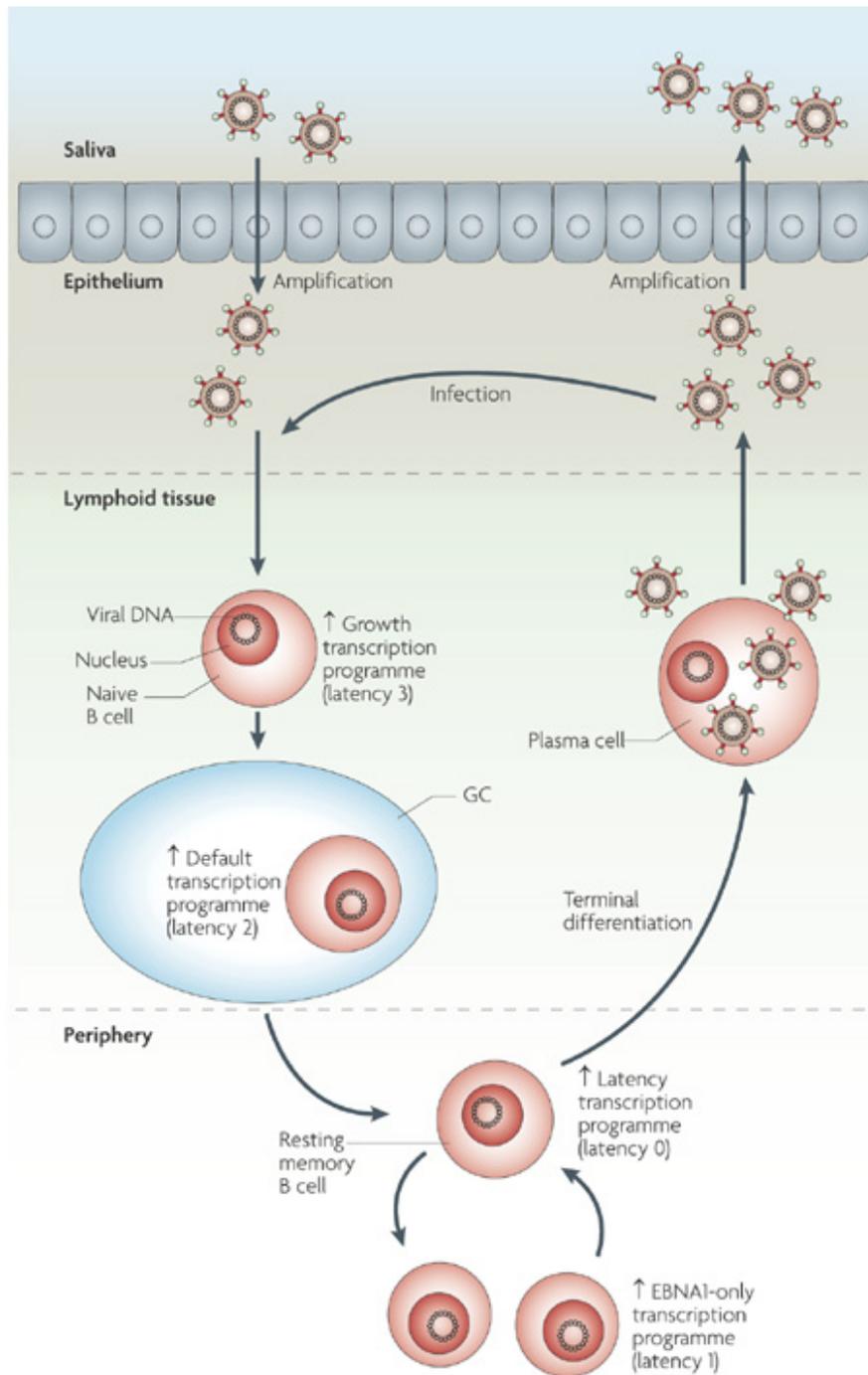
EBV's life cycle can be divided in three different steps: **virus entry and primary infection, latent phase** (composed by latency I, latency II and latency III) and **lytic phase**.

#### **4.1 Virus entry and primary infection:**

EBV is able to infect B lymphocytes and epithelial cells causing a latent infection in most of the cases. In order to latently infect resting memory B cells, the virus uses the normal pathway of B cell differentiation into antibody-secreting B cells (**Figure 2**). This means that mature, naive or memory B cells present in the lymphoid tissue has to be activated and consequently to proliferate in order to become a resting memory B cell which will be able to circulate in the peripheral blood. This mechanism was demonstrated *in vivo* by the group of DA. Thorley-Lawson in 1998<sup>23</sup>. They proved that resting memory B cells circulating through the peripheral blood are the only cells able to undergo spontaneous outgrowth, thus having the possibility of being immortalized.

The entry of EBV into resting B cells or epithelial cells involves the joint action of at least five viral proteins. First, the envelope protein gp350 binds to CD21 (complement receptor) which is present on B cells membrane or to an alternative receptor on epithelial cells which anchors the virus to the target cells. This action allows the binding of gp42 to the corresponding HLA class II molecule, which then mediates the membrane fusion that is carried out by the viral proteins gB, gH and gL<sup>24</sup>. gH and gL glycoproteins are present in all herpesviruses and are necessary for virus penetration into target cells. gH is essential for membrane fusion while gL acts as a chaperone for the proper folding of gH. Virions that contain gH-gL-gp42 complexes infect B lymphocytes whereas virions containing only gH-gL complexes infect epithelial cells.

The group of JM. Palefsky demonstrated that primary infection of epithelial cells from the oropharynx is a complex mechanism that can occur in two different ways: either EBV-infected cells from saliva infect other cells *via* cell-to-cell contact involving the apical cell surface, or EBV virions infect directly the basolateral surface of cells with the joint action of cellular integrins<sup>25</sup>.



**Figure 2** - Scheme explaining the mechanism by which EBV is able to successfully infect and persist lifelong in host cells. (Figure extracted from <sup>26</sup>)

EBV first makes contact and infects epithelial cells where it initiates a lytic program causing an amplification of the viral particles. Then, the virus infects naive B cells present in lymphoid tissues. These activated B cells undergo the latency III program. In order to escape from this program the family of EBNA3 latent genes negatively regulates this program, allowing the infected B cells to migrate and start a germinal center (GC) reaction where it establishes the latency II program. This latency program allows the cell to exit from the GC center and thus to become a resting memory B cell in the periphery. This program is called latency 0 and is characterized by the switch off of all latent genes. When this cell enters in S phase it expresses the EBNA1-only program (latency I) in order to maintain the viral episome.

## **4.2 EBV latent infection:**

During latency, EBV has a strict gene expression pattern which includes: six EBV nuclear antigens (EBNAs), two latent membrane proteins (LPMs), EBV-encoded small RNAs (EBERs) and miRNAs BART (*Bam*HI-A region rightward transcribed). Depending on the proteins that are expressed, EBV latent infection can be classified in three different classes: latency I, latency II and latency III (**Table 2**). All of them have in common the expression of EBNA1.

### 4.2.1 EBV latency programs:

#### -Latency I, the EBNA1-only program:

This latency program is characteristic of the Burkitt lymphoma and implies the expression of a single viral protein, EBNA1, and small non-coding RNAs called EBERs. Nevertheless, there are some controversies because some groups found that the latent membrane proteins 1 and 2 (LMP1, LMP2) are also expressed in latency I cells. This latency I program is the expression pattern of EBV-carrying resting memory B cells which are EBV long-term hosts.

#### -Latency II, the default program:

The latency II program is characteristic of the Hodgkin's lymphoma and the nasopharyngeal carcinoma. Besides EBNA1, latency II cells also express three additional viral latent antigens: LMP1, 2A and 2B.

#### - Latency III, the growth program:

The latency III program is characterized by the complete expression of all EBV latent genes including all the genes expressed in the other latency programs as well as EBNA2, EBNA-LP, EBNA3A, EBNA3B and EBNA3C. This type of latency is observed in infectious mononucleosis (primary infection), LCL's (lymphoblastoid cell lines) and some BL.

#### 4.2.2 EBV latent proteins and gene products

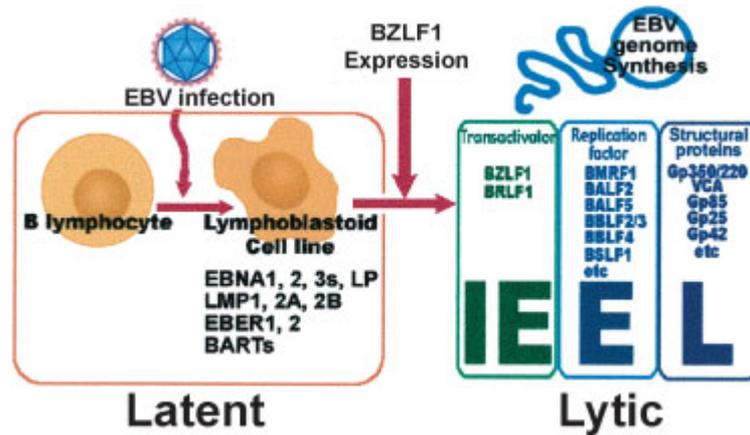
In this paragraph EBV proteins and non-coding RNAs produced during the latent phase will be briefly presented (**Figure 3 and Table 2**):

**EBNA1** (EBV nuclear antigen 1) is the only viral protein expressed all along EBV life cycle. During the latent cycle EBNA1 has three essential functions for EBV: the replication and the maintenance of the viral genome<sup>27</sup> as well as the immune evasion of infected cells (see chapter 2). EBNA1 is also able to inhibit the spontaneous virus reactivation by inhibiting ZEBRA transcription, ZEBRA being essential to activate the lytic program<sup>28</sup>. In addition, EBNA1 is able to interact with premyelocytic leukemia proteins (PML) and induce their degradation, thereby preventing p53 activation and thus induction of apoptosis upon DNA damage<sup>29</sup>. Furthermore, EBNA1 contributes to the oncogenic process by inducing the apoptosis suppressor protein Survivin in EBV-associated lymphomas<sup>30</sup>.

**LMP1** is a transmembrane protein that is expressed in all forms of HL, NPC and LCL. It contains six transmembrane domains that mediate its association with lipid rafts and signaling from two transformation effector sites that are necessary for EBV-mediated B cell transformation. LMP1 mimics the functions of CD40 receptor in B cells which is involved in the regulation of antibody production<sup>31,32</sup>. The group of E. Kieff demonstrated in 1993 that LMP1 is sufficient for EBV-mediated B cell transformation *in vitro*<sup>33</sup>. It is the main EBV-encoded oncogene and it is able to activate the signaling pathways of NF- $\kappa$ B, PI3K, JNK and p38<sup>33,34</sup>. The activation of PI3K kinase pathway promotes cell survival and suppression of apoptosis, while the activation of JNK pathway induces cell proliferation and transformation<sup>35,36</sup>. In epithelial cells, it has been shown that LMP1 activates vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), cyclooxygenase-2 (COX-2) and matrix metalloproteinase 9 (MMP-9). All these factors are involved in the formation of new blood vessels that can feed tumor cells<sup>37,38</sup>.

**LMP2A and LMP2B** are two closely related EBV-encoded proteins expressed during the latency II and III programs. LMP2A contains 12 transmembrane domains (TM) and an N-terminal domain involved in cytoplasmic signaling. On the other hand, the isoform 2B has the same TM domains but lacks the N-terminal functional domain<sup>39</sup>. In B cells, LMP2A prevents the recruitment of the B cell receptor (BCR) in lipid rafts, which abrogates BCR functions. It also promotes B cell growth and induces lymphoma due to a BCR-like signal through the constitutive activation of the extracellular

ERK/MAPK pathway<sup>40</sup>. In epithelial cells LMP2A stimulates cell motility and diffusion and enhances the transition to NPC due to the induction of the metastatic tumor antigen (MTA1)<sup>41</sup>.



**Figure 3-** Epstein-Barr virus life cycle. Figure extracted from<sup>42</sup>.

EBV infects resting B cells inducing a continuous cell proliferation. The resulting lymphoblastoid cells (LCLs) express the full set of latent proteins, which are able to reactivate quiescent B cells to enter into S phase, continue to proliferate and avoid apoptosis. The expression of the two intermediate-early lytic genes BZLF1 and BRLF1 triggers the activation of viral early gene expression involved in DNA replication and metabolism, followed by the expression of the late genes which are mostly involved in the capsid formation.

**EBNA2 and EBNA-LP.** These two proteins are involved in B cell transformation through the activation of the transcription of viral and cellular genes involved in cell transformation. EBNA2 is a phosphorylated DNA binding protein which is able to activate the transcription of CD23, CD21, c-Myc, c-fgr, BLR2/EBI1 and all latent genes<sup>43</sup>. EBNA2 contributes to cancer progression since it is also able to enhance the expression of the anti-apoptotic proteins Bcl-xL, Bcl-2 and bfl-1. Several potential functions like mediating apoptosis have been attributed to EBNA-LP; nevertheless, the most widely confirmed is its ability to act as a coactivator of EBNA2. E. Kieff *et al* demonstrated that EBNA-LP plays a critical role in B cell transformation by stimulating EBNA2-mediated transcription activation<sup>44</sup>.

**EBNA3A, EBNA3B and EBNA3C.** The expression of these three proteins which share a similar structure is under the control of the same promotor. All of them are able to regulate the transcription of EBNA2 and Notch *via* direct binding to RBPJ transcription factor<sup>45</sup>. EBNA3A and EBNA3C are essential for B cell transformation and immortalization whereas EBNA3B is dispensable. The group of F. Wang demonstrated that EBV-infected cells lacking EBNA3B are still able to stimulate B cell transformation and immortalization *in vitro*<sup>46</sup>. EBNA3A and 3C inhibit the production of BIM

transcripts which impedes the apoptosis process and thus allows the proliferation of cancer cells. It has been demonstrated that EBNA3C can affect p53-mediated activities. For example, EBNA3C is able to interact directly with the N-terminal domain of p53 which is also the binding site for several important cellular factors like pRb and c-Myc. In line with this, EBNA3C recruits MDM2 in order to favor p53 ubiquitination and degradation by the proteasome <sup>47</sup>.

**EBERs (Epstein-Barr-encoded small RNAs).** These are EBV-encoded small non-polyadenylated non-coding RNAs which are transcribed by the host RNA polymerase III. EBER1 is 167-nucleotides long whereas EBER2 is 172-nucleotides long. They are the most common non-coding EBV RNAs during the latent phase.

The expression of EBERs enhances tumor formation and growth both *in vitro* and *in vivo* <sup>48</sup>. It has been demonstrated that EBERs are able to interact with La protein, the ribosomal protein L22, phosphoribulokinase (PKR) and other cellular proteins. It has been shown that the association of EBERs with La protein causes a reduction of RNA pol III transcripts stability <sup>49,50</sup>. The binding of EBERs to L22 causes the delocalization of L22 to the nucleoplasm which affects the translation of specific RNAs. Finally the interaction of these RNAs with PRK protein, which is involved in interferon regulation, blocks the PRK-mediated phosphorylation of the initiation factor 2 $\alpha$  (IF2 $\alpha$ ), which in turn blocks the translation initiation mediated by IF2 $\alpha$  and also the resistance to IFN $\alpha$ -induced apoptosis <sup>51</sup>.

**EBV-encoded miRNAs BARTs (BamHI A rightward transcripts) and BHRF1 (BamHI H rightward ORF1).** EBV's miRNAs are encoded from two different regions of EBV genome: BARTs from the BamHI A region rightward transcript and BHRF from the 5' and 3'-UTRs of BamHI H rightward ORF1. BART region encodes 40 different miRNAs whereas BHRF region encodes 4 other miRNAs.

BARTs miRNAs are present in all EBV-associated latency phases and their principal function is to prevent B and epithelial cells to trigger apoptosis. For example, miR-BART5 promotes cell survival by targeting the pro-apoptotic gene PUMA and, in NPC, BARTs miRNAs contribute to the establishment of the latent infection.

On the other hand BHRF1 expression is restricted mostly to latency III program <sup>52</sup> and accomplishes similar functions.

**Table 2**-EBV latency programs. The different viral products expressed during each latency phase and the diseases associated to each programs. Table extracted from <sup>42</sup>.

| Latency                   | Main Viral Genes Expressed <sup>a</sup>                               | EBV Infection Context   |
|---------------------------|---|---|
| Type I (EBNA1-only)       | EBERs, BARFO, EBNA1, (LMP2A)  | <ul style="list-style-type: none"> <li>• Proliferating memory B cells and BL cells <i>in vivo</i></li> <li>• EBV-associated gastric carcinoma<sup>b</sup></li> </ul>  |
| Type II (Default program) | EBERs, BARFs, EBNA1, LMPs 1, 2A, and 2B                               | <ul style="list-style-type: none"> <li>• Germinal center B cells</li> <li>• Neoplastic cells from NPC and EBV-positive classical HL</li> </ul>  |
| Type III (Growth program) | EBERs, BARFs, EBNA1s (1, 2, 3A, 3B, 3C, and LP), LMPs (1, 2A, and 2B) | <ul style="list-style-type: none"> <li>• Naïve B cells and lymphoblastoid cell lines</li> <li>• Cells from lymphoproliferative diseases arising in immunosuppressed hosts (e.g., PTLD and immunoblastic NHL of the central nervous system)</li> </ul> |

Once a B cell is immortalized it usually maintains EBV in a latent phase. However virus life cycle can switch from latency to replication spontaneously or *via* induction by external stimuli like 12-O-tetradecanoylphorbol-13-acetate (TPA) and butyrate <sup>53</sup>. Later on, it was shown that the expression of the single viral gene BZLF1 (also known as ZEBRA) can trigger the lytic cycle.

#### 4.3 EBV lytic cycle

The lytic cycle is the active phase of EBV life cycle in which approximately 80 viral proteins are expressed in order to produce the maximal quantity of virions that will further infect other cells. The lytic phase is divided in three distinct steps among which various proteins are expressed: the immediate-early, early and late proteins. The expression of ZEBRA, the first lytic protein, triggers the switch from latent to lytic phase (**Figure 3**). ZEBRA is a transcription factor that controls the expression of the immediate-early gene Rta that consequently, and together with ZEBRA, will control the expression of many early genes <sup>4</sup>. ZEBRA activates the transcription by binding to ZEBRA response elements (ZREs) found in the promoters of EBV lytic cycle target genes. During the early phase EBV encodes genes that are involved in the nucleotide metabolism like the DNA polymerase or the thymidine kinase BXL1. Finally during the late phase the “core replication” genes are expressed in order to ensure a fast replication of the viral genome. Among these genes are BMRF1 (DNA polymerase processivity factor), BBLF4 (helicase), BALF5 (DNA polymerase), BSLF1 (primase), BALF2 (single-stranded DNA binding protein) and BBLF2/3 (primase accessory protein) <sup>54</sup>.



## CHAPTER II: EBV, a master in the art of hiding

The presentation of antigenic peptides by Major Histocompatibility Complex (MHC) class I and II molecules allows the immune system to distinguish between self and non-self. Thanks to this mechanism, the immune system is able to detect bacterial, parasite or viral infections. This process involves the degradation of polypeptides into peptides and their later association with MHC molecules in the endoplasmic reticulum (ER). These loaded MHC complexes are then transported to the cell surface of the antigen presenting cell (APC) and recognized by a specific T cell receptor (TCR) which is exposed at the surface of T cells. There are two different sources of antigenic peptides: intracellular antigens and extracellular antigens. The presentation of intracellular peptides (produced by the cell machinery) is driven by MHC class I molecules that are recognized by cytotoxic T lymphocytes (CTL, T CD8<sup>+</sup>). On the other hand, extracellular peptides (not produced by the cell) are presented to T-helper lymphocytes (T CD4<sup>+</sup>) by the MHC class II molecules.

In normal conditions MHC class I molecules present peptides derived from endogenous proteins in all nucleated cells. Nevertheless, if a viral infection occurs, viral antigens produced by the infected cells will be processed by the 26S proteasome and small peptides of about 8-10 residues will be shuttled by the transporter associated with antigen processing (TAP, 1 and 2) into the ER where the assembly with the MHC class I molecule will take place <sup>55</sup>.

EBV has evolved several strategies to avoid immune recognition and destruction of infected cells during both lytic and latent phases. These strategies are detailed below.

### 1. EBV immune evasion strategies during lytic phase

Gammaherpesviruses have evolved several strategies so that infected cells can evade the host immune system. In this section, EBV strategies that allow infected cells to evade T cell recognition during the lytic phase /virion production phase will be described.

### ***1.1 Strategies against the activation of the IFN- $\gamma$ pathway***

The interferon  $\gamma$  (IFN- $\gamma$ ) is one of the most important cytokines released in response to all viral infections and to some bacterial and protozoal infections. It is expressed during the innate response by natural killer cells (NK) and by T CD8<sup>+</sup> and T CD4<sup>+</sup> Th1 cells during the adaptive response. This cytokine induces a cascade of signaling that leads to anti-viral responses targeting all steps of the life cycle of the virus. It is an activator of macrophages and it also enhances the expression of MHC class II molecules. Because of this,  $\gamma$ -herpesviruses have developed several strategies in order to inhibit either IFN- $\gamma$  production or its signaling pathways. In particular, during the lytic phase, EBV expresses several antigens like BCRF1 (which is a homolog of IL-10), BZLF1 and BARF1 (vCSF). BCRF1 encodes vIL-10, a virokine that mimics the immune suppressive action of IL-10. BCRF1 is expressed during the late lytic phase and causes the inhibition of IFN- $\gamma$  and IL-2 synthesis and also inhibits the maturation of dendritic cells<sup>56</sup>. BZLF1 is expressed during the intermediate early lytic phase and encodes ZEBRA which is the trigger of the lytic phase, as described above. BZLF1 blocks tyrosine phosphorylation and nuclear translocation of STAT1 (a transcription factor which is activated by IFN- $\gamma$ ), decreases IFN- $\gamma$  expression and also inhibits the MHC class II surface expression which is stimulated by IFN- $\gamma$ <sup>57</sup>. Finally, BARF1 is expressed during the early lytic phase and prevents the proliferation of macrophages and also inhibits IFN- $\gamma$  production by monocytes<sup>58</sup>.

### ***1.2 Strategies against MHC class I antigen presentation pathway***

$\gamma$ -herpesviruses also encode other proteins which can affect several steps of the MHC class I antigen presentation pathway. In the case of EBV, the BCRF1 gene which is expressed during the lytic phase is able to downregulate the expression of the proteasome subunit LMP2 and of TAP1, thus preventing the production and presentation of virus-derived antigenic peptides<sup>59</sup>. Moreover, BGLF5, an EBV-encoded alkaline exonuclease, can promote host mRNA degradation by retaining mRNAs in the nucleus and thereby shutting off protein synthesis. The resulting inhibition of cellular protein synthesis serves to ensure maximal viral gene expression and evasion from host immune response, as it induces host MHC class I expression shutoff<sup>60</sup>. The early lytic expressed BNLF2 $\alpha$  gene is able to interact with the TAP transporter and to inhibit the binding capacity of TAP to both the antigenic peptides and to ATP. Given that ATP hydrolysis is essential to ensure the opening of the transmembrane pore and the translocation of the peptides to the ER this also participates to immune evasion of  $\gamma$ -herpesviruses<sup>61</sup>. Finally, during the early stage of the lytic cycle a third gene *BILF1* also

contributes to the attenuation of MHC class I responses. *BILF1* is a G-protein-coupled receptor (GPCR) which is able to increase MHC class I molecules turnover by inducing MHC class I internalization from the cell surface and its subsequent degradation by the lysosomal compartment <sup>62</sup>.

### *1.3 Strategies against MHC class II antigen presentation pathway*

The antigenic presentation by the MHC class II molecules involves the loading of free MHC class II molecules inside the MHC II loading compartment (MIIC) with antigenic peptides produced from the endocytic pathway. Once loaded, MHC class II complexes are translocated to the surface of APC cells. In contrast to the MHC class I antigen presentation pathway, little is known about how  $\gamma$ -herpesviruses manage to escape T CD4<sup>+</sup> response.

The intermediate early lytic BZLF1 gene has the ability to inhibit the transcription of the gene encoding MHC class II molecules by binding and repressing CIITA PIII. CIITA gene is a master regulator of the MHC class II pathway that promotes the expression of MHC class II molecules. This gene is under the control of several promoters, the number I and III being the most important in B cells in order to ensure the constitutive expression of CIITA <sup>63</sup>.

Interestingly, EBV uses the MHC class II complex as a cofactor of CD21 in order to infect B cells <sup>64</sup>. EBV attachment to B cell membrane occurs via the envelope protein gp350 which binds to the CD21 receptor. In order to achieve B cell infection, the interaction of the envelope protein gp42 encoded by BZLF2 is required <sup>24, 65</sup>. gp42 associates to MHC class II molecules in the ER and once at the APC surface it can block the interaction of the TCR with the peptide-MHC class II complexes, thereby inhibiting antigenic recognition by CD4<sup>+</sup> T helper cells <sup>66</sup>. In addition, the early lytic gene *BGLF5*, which encodes the EBV endonuclease, catalyzes the host protein synthesis shutoff by inducing the mRNA degradation of several host genes, among which the MHC class II molecules <sup>60</sup>. M. Rowe *et al* demonstrated that cotransfection of *BGLF5* and GFP vectors in 293 cells reduced the levels of GFP mRNA. Moreover, the half-life of GFP mRNA decreased in the presence of *BGLF5* demonstrating that it affects mRNA turnover.

## 2. Immune evasion strategies during latent phase

Human  $\gamma$ -herpesviruses have evolved a sophisticated strategy to escape T CD8<sup>+</sup> recognition. This strategy is mainly based on the control of the expression of a single viral protein: the genome maintenance protein (GMP). GMP can be considered as the Achilles' heel of these viruses because it is essential for viral genome replication and maintenance, and at the same time it is highly antigenic and T cells raised against these proteins exist in all infected individuals.

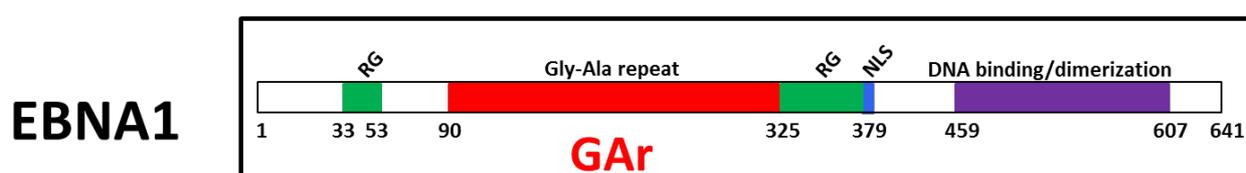
### 2.1 *EBNA1 Genome Maintenance Protein (GMP): the Achilles' heel of EBV*

GMPs are expressed by all  $\gamma$ -herpesviruses. They have the crucial role of ensuring the replication and maintenance of the viral genome after each M phase of the cell cycle. Indeed, they are DNA binding proteins that display two pivotal roles: i) they participate in viral genome replication and ii) they tether the viral episome to host cell chromosomes, thereby ensuring the maintenance of the viral genome in each daughter cell during host cell division<sup>67</sup>. Like all GMPs, the EBNA1 protein, the GMP of EBV, has two essential functions during the viral life cycle: the replication of the viral episome and its maintenance in daughter cells. In this section I will present a description of EBNA1, with a focus on these two GMP functions.

#### 2.1.1 EBNA1 protein organization

EBNA1 is a multifunctional protein encoded by BKRF1 gene which is located in the BamHI-K fragment of the viral episome (**Figure 1**). It is a homo-dimeric DNA-binding protein expressed in all EBV-infected cells<sup>68</sup>. EBNA1 protein from the EBV prototype strain B95.8 is a 641 amino acids protein that contains three different functional domains: a central glycine-alanine rich (GAR) domain, two arginine-glycine domains (RG) and a C-terminal domain<sup>69</sup> (**Figure 4**). The first 30 amino acids of the N-terminal domain of EBNA1 are not associated with any particular cellular function. The first RG-rich domain is located from residues 33 to 89 and the second between residues 328 and 386 and both are responsible for EBNA1 association with host chromosomes<sup>27</sup>. These RG-rich domains are similar to the RGG motif present in several RNA-binding proteins and J. Norseen *et al* demonstrated that they are involved in the recruitment of the origin recognition complex (ORC) to the origin of replication (OriP) of EBV genome for replication initiation<sup>70</sup>. The central Gly-Ala (GAR) domain of EBNA1 is conserved in all

EBV strains and varies in length from 60 to 300 residues <sup>71</sup>. The canonical GAR domain contains 239 residues. This central GAR domain is crucially involved in EBNA1-based EBV immune evasion during the latent phase of viral infection. Of note, this ability of EBV to evade the immune system depends on the length of GAR: the longest GAR display the most efficient immune evasion. The C-terminal domain of EBNA1 is a DNA binding domain (DBD). EBNA1 DBD binds to the Qp promoter which is responsible for EBNA1 transcription during latency phases I and II <sup>72</sup> (**Figure 4**). EBNA1 DBD also interacts with the OriP of the viral genome at each M phase of the cell cycle to ensure the viral replication and maintenance.



**Figure 4-** EBNA1 protein organization.

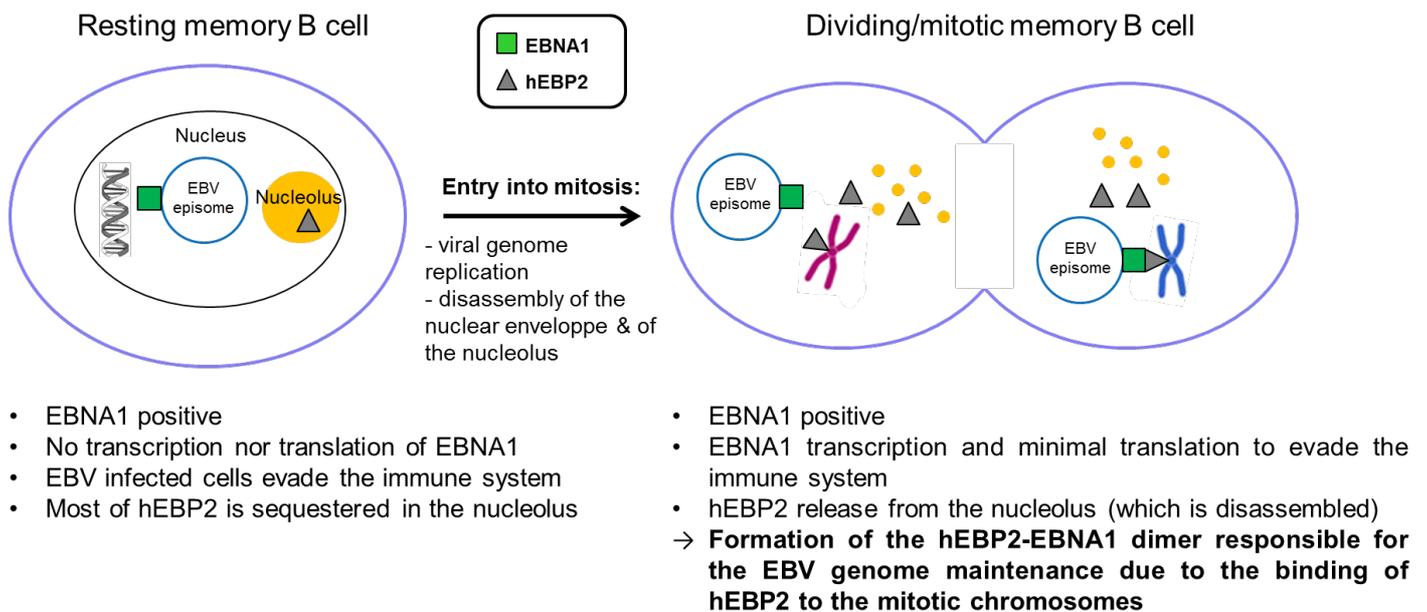
All EBNA1 domains are represented, as well as the amino acid positions. In green are represented the two glycine-arginine (RG) domains, in red the glycine-alanine repeat (GAR), in blue de nuclear localization signal (NLS) and in violet the DNA binding domain (DBD).

### 2.1.2 EBNA1 function in genome replication and maintenance

As described above, both EBV episome replication and maintenance rely on EBNA1 binding to multiple sites on the OriP. The OriP covers approximately 1700 bp and contains two different *cis*-acting elements that are both necessary to ensure episomal replication. The first element is a family of 20 tandem copies of a 30 bp sequence (FR) and the second is a 114 bp dyad symmetry (DS) <sup>73,74</sup>. FR elements are not directly involved in DNA replication but rather in mitotic segregation and transcriptional activation. The DS element is responsible for DNA replication and it has been shown to be sufficient for EBV replication in the presence of EBNA1 <sup>75</sup>. The crystal structure of EBNA1 bound to DNA has been solved and it shows that its binding site is composed of two different DNA binding domains. The N-terminal DNA binding domain involves the residues 470-503 of EBNA1 and is called the flanking domain, whereas the C-terminal one, called the core domain, includes residues 504-604

<sup>76</sup>. Structure resolution shows that EBNA1 binds to its binding site as a dimer. The core domain possesses two  $\alpha$ -helices that make specific contacts with the DNA.

Moreover, EBNA1 assures viral genome maintenance by tethering the viral episome to the host mitotic chromosomes. EBNA1 is the most studied EBV-encoded protein and, at the end of the nineties, the team of L. Frappier used EBNA1 as a bait in a two-hybrid screen to identify human proteins that interact with EBNA1. EBP2 (EBNA1 binding protein 2) was shown to specifically interact with EBNA1 <sup>77</sup>. EBNA1 region that binds to EBP2 has been mapped to the small central arginine-glycine (RG) rich domain. The deletion of this RG domain in EBNA1 prevents its interaction with EBP2 and consequently the ability of EBNA1 to support the long-term maintenance of a plasmid containing the EBV origin (OriP) in human cells (**Figure 5**).



**Figure 5-** The GMP of EBV, EBNA1, assures the replication and maintenance of the viral genome on every S phase of the cell cycle. Figure extracted from <sup>2</sup>.

Resting memory B cells are EBNA1 positive but there is not transcription neither translation of EBNA1. In contrary, mitotic B cells expressed a minimal level of EBNA1 in order to assure episomal maintenance which is low enough to be undetected by the host immune system.

EBNA1 is thus an essential protein for EBV genome to persist into highly dividing cells. It is also highly antigenic and CD8<sup>+</sup> T cells raised against EBNA1 exist in all infected individuals which may compromise EBV persistence in its host. However EBNA1-infected cells are not eliminated by the host

immune system. This is due to another property of EBNA1 that allows EBV infected cells to avoid T cell recognition.  $\gamma$ -herpesviruses have indeed developed several strategies in order to decrease the quantity of antigenic peptides derived from their GMPs that are presented at the cell surface of infected cells *via* the MHC class I complex. Most  $\gamma$ -herpesviruses strategy to evade the immune system recognition during latent phase is based on a tight control of their GMP expression level: it should be high enough to ensure genome replication and maintenance but low enough to avoid the detection of virus-infected cells by T CD8<sup>+</sup> cells.

In summary, most EBV strategy to evade the host immune system is to tightly control EBNA1 level to a minimal level to ensure viral genome replication and maintenance and at the same time to minimize presentation of EBNA1-derived antigenic peptides by the MHC class I complex<sup>74</sup>. As described in the next section, EBV strategy to evade the immune system lays on the GAR domain of EBNA1.

## *2.2 EBNA1 GAR domain is responsible for EBNA1-based immune evasion*

As state above, most EBV's strategy to evade the immune system is based on a tight control of its GMP (EBNA1) expression level. This tight control actually involves its central GAR domain (**Figure 4**). Although the GMPs of all  $\gamma$ -herpesviruses share poor sequence similarities, they all possess a central domain which sequence is highly repetitive. This domain is not essential for genome replication and maintenance functions but it plays a crucial role in the mechanism of immune evasion of infected cells. The function of this central domain on immune evasion has been shown only for human  $\gamma$ -herpesviruses EBV and KSHV, while for Old / New World Monkeys  $\gamma$ -herpesviruses, this function doesn't seem to exist.

Two mechanisms have been successively explored: GAR was initially thought to act *via* its ability to inhibit proteasome degradation, thereby limiting the production of EBNA1-derived antigenic peptides<sup>77</sup>. It was later demonstrated that its effect rather involves its capacity to self-limit the translation of its own mRNA *in cis*, a mechanism which also results in the reduction of the production of antigenic peptides<sup>78</sup>.

Interestingly, the switch from the first model (26S proteasome inhibition) to the second model (translation inhibition *in cis*) paralleled the evolution of models and knowledge regarding the main source of antigenic peptides<sup>79,80</sup>. These two models will be described below.

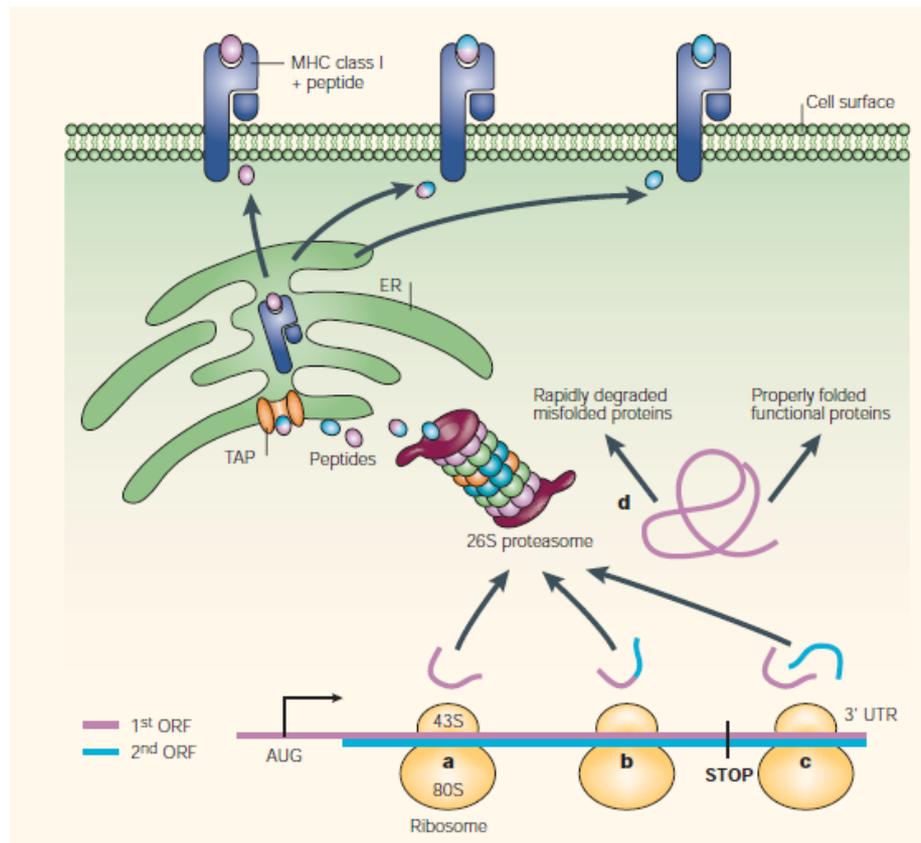
### 2.2.1 EBNA1 GAR domain is responsible for latent EBV-infected immune evasion

In the beginning of the 90's the group of MG. Masucci observed that latently EBV-infected cells evade the immune system despite the fact that CTLs directed toward EBNA1 are present in all EBV-infected individuals. In addition, the GAR domain represents the major target of T cells raised against EBNA1-derived epitopes. For this reason, they wondered if this particular domain could affect the host cell immune response. To answer this question, they constructed several recombinant vaccinia (vacc) viruses expressing the full-length EBNA1 or EBNA1ΔGAR, a form of EBNA1 deleted for its central GAR domain, in which they inserted the antigenic peptide containing the amino acids 416-424 from the EBNA3B protein. Then, they showed that fibroblasts infected with vacc expressing EBNA1 remain undetected from specific CTLs against EBNA3B whereas fibroblasts infected with vacc expressing EBNA1ΔGAR or only EBNA3B epitope were efficiently recognized and eliminated<sup>81</sup>. Moreover, they demonstrated that GAR domain acts as a *cis*-acting element, since the overexpression of EBNA1 alone didn't affect the CTLs recognition of EBNA3B itself. They also determined the ability of EBNA1 GAR domain to act in N-terminal or C-terminal position by introducing the GAR sequence in frame with EBNA3B coding sequence. The results showed similar CTLs recognition of fibroblasts infected with vacc EBNA1 or vacc EBNA3B-GAR, which means that the GAR effect on immune evasion is not significantly affected by its position in N- or C-terminal. The group of MG. Masucci showed that GAR has an inhibitory effect on antigenic recognition but the mechanism involved in this inhibitory effect was still undeciphered. Nevertheless, they proposed two different mechanisms: GAR could interfere with the proteolytic pathway that leads to antigenic peptides production, or GAR-containing proteins could be sequestered in a compartment which is not accessible for antigenic peptides processing<sup>77</sup>.

### 2.2.2 Origin of the antigenic peptides presented by MHC class I molecules

The source of antigenic peptides for MHC class I molecules has been a subject of controversy. Peptides were originally thought to be produced through the degradation of full proteins in the cytoplasm by the 26S proteasome. This hypothesis was supported by several observations: i) blocking the proteolysis in the endosomes or lysosomes did not affect the antigenic presentation of MHC class I molecules, while antigenic presentation by class II was affected, ii) drugs able to block the activity of the proteasome also inhibit the antigenic presentation by MHC class I<sup>82</sup>. Moreover, proteins that are specifically targeted to the cytoplasm are also presented by the MHC class I<sup>79</sup>, iii) the last and most important observation was that cells lacking the TAP transporter were not able to present

antigenic peptides by MHC class I, since they lack the continual supply of antigenic peptides from the cytoplasm<sup>82</sup>. However, the observation that viral proteins are in general very stable suggests that the degradation of full-length proteins by the proteasome and the later presentation of derived antigenic peptides by the MHC class I would take many hours or even days. Nevertheless, T CD8<sup>+</sup> responses against viral antigens are driven in only one hour. This can be explained only if there is an alternative mechanism that provides antigenic peptides more rapidly.



**Figure 6-** Defective ribosomal products (DRiPs) model. Figure extracted from<sup>80</sup>.

The source of antigenic peptides in the MHC class I pathway has been a subject of controversy. It was thought that the majority of antigenic peptides comes from the degradation of full-length proteins by cytosolic proteasome degradation and their subsequent translocation into the endoplasmic reticulum in which they are subsequently loaded onto MHC class I molecules. Later it was proposed that the most important source of antigenic peptides comes from defective products of the mRNA translation (DRiPs). These DRiPs are produced: a) by alternative initiation or pre-termination, b) by ribosomal frameshift, c) from the 3' non translated region or d) from misfolded proteins.

In 1996 another source of antigenic peptides was proposed. The most important source of antigenic peptides was suggested to be the defective ribosomal products (DRiPs) that are products generated during the first round of translation<sup>83</sup>. These DRiPs include defective, misfolded or pre-mature

polypeptides as a result of mistakes during the translation process (**Figure 6**). The first observation that supported this model came from E.A Reits *et al* in 2000. They were trying to find the source of the antigenic peptides presented by TAP to the MHC class I. Because TAP mobility increases when it is not associated to an antigenic peptide, this can be used to monitor the intracellular peptide content *in vivo*. They demonstrated that TAP mobility in the ER membrane decreases when it is associated to peptides. Then, they showed an increase in TAP mobility after cycloheximide treatment in cells infected with influenza A virus. This allowed them to conclude that the most important source of antigenic peptides comes from mRNA translation <sup>84</sup>. At the same time, U. Schubert *et al* demonstrated that cycloheximide treatment inhibits the transport of MHC class I molecules from the ER to the cell surface. They showed that one-third of newly synthesized proteins are rapidly degraded and this seems to be the source of MHC I peptides <sup>85</sup>.

Most of the experiments that favor the DRiPs hypothesis are based on the observation that presentation by MHC I molecules is markedly reduced when *de novo* protein synthesis is inhibited. This theory has been shown to be right for most proteins but not all, since it assumes that all mature proteins are accessible for MHC I presentation, and in fact antigens that result from autophagy or lysosomal degradation would escape from proteasome degradation and thus would never achieve MHC class I loading. Moreover, protein synthesis inhibitors, like cycloheximide, not only affect the synthesis of antigenic peptides but also could affect the production of several proteins involved in the MHC class I pathway, and thus cycloheximide-based experiments cannot be used as a fully convincing demonstration that DRiPs are the major source of antigen peptides. J.D Colbert *et al* challenged the DRiPs model by studying the proportion of mature proteins to MHC I presentation using a tetracycline-inducible system to express antigens that are conditionally stabilized upon ligand binding (Shield-stabilized FKBP antigen). Results showed that at least for this particular antigen the major source of antigenic peptides for MHC I pathway seems to be mature proteins since DRiPs are supposed to be derived from defective non-functional proteins, and in contrast Shield-stabilized Ags bind ligands and are fluorescent. Also, DRiPs are normally and rapidly polyubiquitinated; however polyubiquitination was not observed for this particular antigen <sup>86</sup>. Finally they showed that inhibition of protein synthesis indeed blocks the synthesis of other components of the MHC I pathway.

### 2.2.3 Identification of the mechanism by which GAR domain allows immune evasion: proteasome degradation or translation inhibition?

As stated above, the main source of antigenic peptides has been largely debated, between the full-length protein degradation model and the DRiPs model. Interestingly, the role of GAR in EBNA1 immune evasion followed the same evolution.

It was initially thought that the inhibition of the antigenic presentation by the GAR domain was due to inhibition of protein degradation by the 26S proteasome, which was fully consistent with the model existing at that moment, establishing that the principal source of antigenic peptides comes from degradation of full proteins by the 26S proteasome. Several studies showed that the fusion of the GAR domain to various instable proteins led to the inhibition of their degradation by the 26S proteasome<sup>87, 88</sup>. In line with this theory, J. Levitskaya *et al* demonstrated in 1997 that, indeed, *in vitro* translated EBNA1 was resistant to proteasomal degradation whereas a form of EBNA1 which lacks its GAR domain, EBNA1ΔGAR, was sensitive and efficiently degraded. Also EBNA3B chimeras containing full-length GAR sequences or 17 Gly-Ala residues were not degraded by the 26S proteasome. Nevertheless, they were not able to determine which step of the degradation pathway was affected, since they showed that GAR containing proteins could be efficiently polyubiquitinated<sup>89</sup>. They thus proposed that the GAR sequences could modify the folding pattern of the resulted peptides and that this may affect their association with components of the ubiquitin-proteasome pathway. This was supported by the observation that GAR sequences inserted in the IκB protein allows the polyubiquitination of the protein but prevents the formation of stable complexes with the 26S proteasome<sup>87</sup>. Based on these results the GAR polypeptide has been proposed to form secondary β-sheets structures which are resistant to unfolding and thus block the binding of the proteasome complex. Another important aspect is the GAR-length dependency: the longer the GAR sequence is, the strongest is its effect on proteasomal inhibition.

Three years later J. Tellam *et al* demonstrated that forcing EBNA1 to intracellular degradation by combining the N-rule and the ubiquitin (Ub) pathways, dramatically reduces EBNA1 half-life. Ub-EBNA1 constructs were efficiently degraded by the proteasome and the resulting antigenic peptides were actively presented to CTLs. These results were the first demonstration that the GAR domain is not completely able to inhibit proteasomal degradation<sup>90</sup>.

However, it was later demonstrated that, contrary to J. Levistkaya observations, the deletion of the GAR domain doesn't affect EBNA1 turnover, as it is a highly stable protein by itself<sup>78</sup>. Concomitantly, observations showed that the inhibition of translation of a stable protein decreases the antigenic presentation which supports the idea of an alternative source of antigenic peptides.

At the same time, JW. Yewdell *et al* indeed demonstrated that the so-called DRiPs (defective ribosomal products) which are abortive products of translation, are the main source of antigenic peptides<sup>83, 85</sup>.

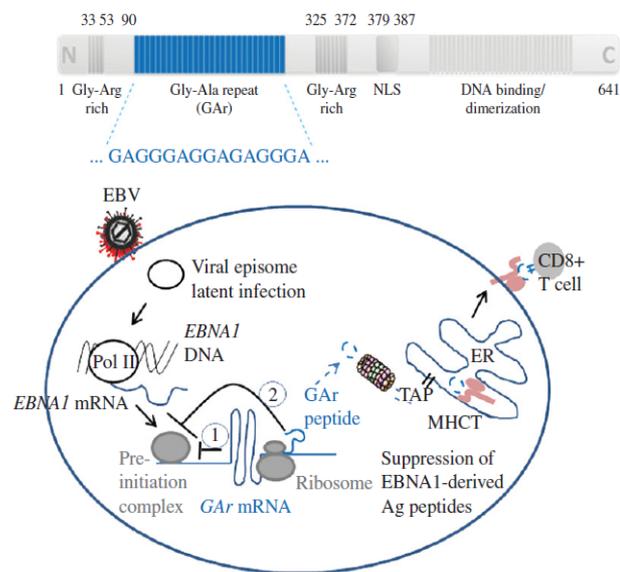
Soon after it was proven that the GAR domain effect was in fact due to the inhibition of translation of its own mRNA *in cis*<sup>80, 91</sup>. In line with this demonstration was the observation that the truncated EBNA1ΔGAR protein is normally translated and leads to an efficient presentation of EBNA1-derived antigenic peptides and thereby recognition of EBNA1-expressing cells by specific T cells. This constitutes the proof of principle that the GAR domain of EBNA1 acts by inhibiting the translation of its own mRNA, thereby limiting the production of DRiPs and thus the presentation of EBNA1-derived antigenic peptides. Later, S. Cardinaud *et al* demonstrated that the effect of the GAR domain was dependent on its position inside the mRNA<sup>92</sup>. They made two different constructs of EBNA1 mRNA in which they inserted the ovalbumin antigen peptide SIINFEKL (SL8) upstream or downstream of the GAR domain. *In vitro* translation experiments showed that both constructs had the capacity of inhibiting EBNA1 translation, and that this inhibition was due to an overall translation inhibition of EBNA1 mRNA and not to difficulties in ribosome binding. In addition, they demonstrated that when the SL8 was present upstream the GAR domain, T CD8<sup>+</sup> against SL8 normally recognized this antigenic peptide, whereas when it was located downstream the GAR, the translation of SL8 was strongly reduced, as was antigenic presentation<sup>92</sup>. These data suggest that the GAR domain efficiently inhibits the translation of its own mRNA when it is fused to the N-terminal of the fusion protein.

### 2.3 Mechanism of GAR-based translation inhibition

Nowadays most of the people working in the EBV field acknowledge that most, if not all, of GAR-based effect on immune evasion is due to its ability to self-inhibit the translation of its own mRNA *in cis*. The exact mechanism of this self-inhibition of translation was under investigation and largely debated and was still undechiphered at the time I started this thesis.

Two non-exclusive hypotheses have been proposed: (i) the inhibition of translation by the GAR domain could be due to the GAR polypeptide itself <sup>78, 79</sup>; (ii) the particular structure of the GAR domain encoding mRNA could be responsible for its effect <sup>93</sup>. These two mechanisms are not mutually exclusive and several observations demonstrated that probably both of them are required for EBV to efficiently self-limit EBNA1 expression and therefore evade the immune system during latent infection (**Figure 7**).

These two hypotheses will be presented and discussed in the next section.



**Figure 7-** EBV's EBNA1-based mechanism of MHC class I immune evasion.

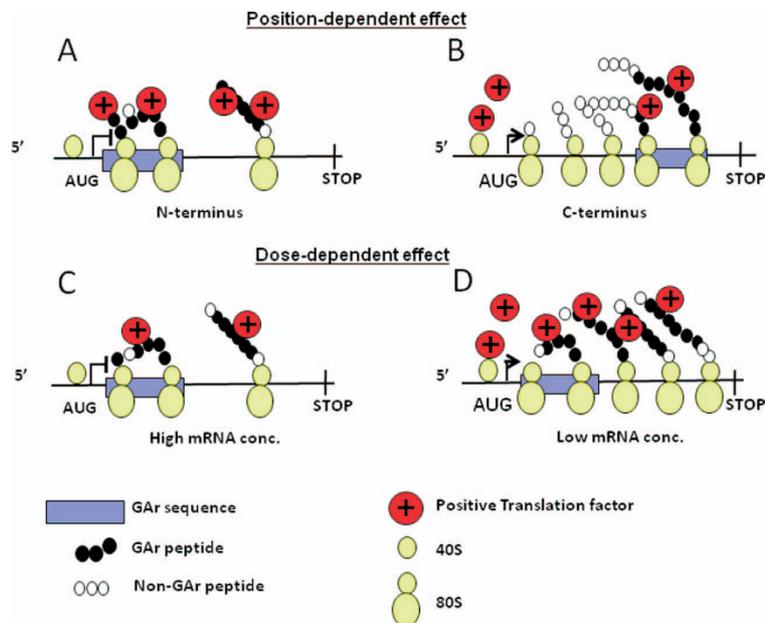
EBNA1 protein is the only protein expressed in all EBV-infected cells. It's central Gly-Ala domain (GAR) inhibits its own translation *in cis* which causes a decrease in the production of antigenic peptides and thus a decrease in T CD8<sup>+</sup> recognition. The mechanism involved in this translation regulation is still undechiphered, however it has been suggested to involve either 1) the structure of GAR-encoding mRNA or 2) the GAR polypeptide itself, or both. Figure extracted from <sup>4</sup>.

### 2.3.1 *GAr polypeptide is responsible for the translation inhibition of its own mRNA.*

This first model establishes that the nascent GAr polypeptide would inhibit the initiation of translation of its own mRNA *in cis*. S. Apcher *et al* demonstrated that the insertion of c-myc IRES (internal ribosomal entry site) in the 5'-UTR of EBNA1 mRNA completely abrogates GAr-based inhibition of translation and antigenic presentation<sup>94</sup>. Since this insertion did not modify the mRNA coding sequence nor the protein sequence, this result suggests that the GAr-based inhibition of translation requires cap-dependent translation initiation factors. Nevertheless, this effect was only observed for the c-myc IRES and not for other viral IRES like EMCV (encephalomyocarditis virus). This could be explained since many authors showed that c-myc IRES can activate the cap-independent translation of nuclear mRNAs thanks to a collaboration with some nuclear factors, which allows mRNA export to the cytoplasm. In contrast, EMCV IRES can only activate the translation of cytoplasmic mRNAs<sup>95</sup>. This suggests that GAr-carrying mRNAs have a nuclear localization in comparison to cellular mRNAs which are generally located in the cytoplasm, what we recently confirmed for EBNA1 mRNA.

In addition, prematurely terminated EBNA1 products were not detected, which supports the idea that the ribosome has no problem to progress through the GAr sequence, and antibodies directed against the GAr domain can indeed enhance translation, which supports the idea of a direct role for the GAr peptide<sup>5, 94</sup>. This polypeptide hypothesis is also reinforced by the fact that other lymphocryptovirus like *Papiine herpesvirus 1* and *Macacine herpesvirus 4* contain a GMP (baEBNA1 and rhEBNA1, respectively) which fulfills the same function than EBNA1 in genome replication and maintenance but which has no effect on immune evasion<sup>67</sup>. Both GMPs present a GAr-like domain which differs from EBNA1 GAr domain in their amino acid composition. Indeed, their Gly-Ala repeats are disrupted by serines (Ser). In line with these observations, replacing some Gly of EBNA1 GAr domain by Ala or Ser stimulates its translation *in vivo*<sup>5</sup>. All these observations indicated that the GAr polypeptide seems to be a key factor in GAr-based translation inhibition. A model in which GAr position effect could be explained by the fact that the nascent GAr polypeptide could interfere with the binding of translation initiation factors has been proposed. Hence, according to this model, when GAr is located in a C-terminal position it cannot efficiently interfere with translation inhibition (**Figure 8**). Moreover, *in vitro* translation experiments showed that GAr-based inhibition of translation is dependent on the quantity of mRNA to be translated. This observation is in line with the presence of

a positive translation factor as at high mRNA concentration the GAR polypeptide / translation factor ratio is high hence allowing an efficient translation inhibition effect. In contrast, if this ratio is low the predominance of the translation factor in comparison to GAR-peptide leads to an inefficient inhibition of translation <sup>5</sup>.



**Figure 8-** Proposed model for GAR-based inhibition of translation. Figure extracted from <sup>5</sup>.

A and B panels show the position-dependent effect of the GAR whereas panels C and D explain the dose-dependent effect observed in an *in vitro* translation assay.

### 2.3.2 GAR-encoding mRNA sequence is responsible for GAR-based translation inhibition

This theory was first supported by observations that the GMPs of other herpesviruses do contain a central domain which has few or even absolutely no amino acid conservation but high homology in terms of nucleotide sequence and purine enrichment (**Table 3**) <sup>96</sup>. For example, in LANA1 (GMP of Kaposi-sarcoma associated virus) the glutamine-glutamate-aspartate (QED) central domain has no homology with GAR protein sequence, but in terms of nucleotide sequence both are purine-rich and present more than 50% homology. Interestingly, introducing a frameshift in QED central domain produces a new repeat domain which has 65% homology with GAR protein sequence <sup>97</sup>. This suggests that the nucleotide sequence encoding the central domain of the GMPs rather than the encoding peptide is involved in GAR-based translation inhibition and that the purine-rich region could be

responsible for the immune evasion by causing the stalling of the ribosomes, and thus pre-termination of the elongation process <sup>98</sup>. This was further supported by observations showing that +1 and +2 frameshifts within the GAR mRNA sequence cause changes in the aminoacid composition of the repetitive sequence (+1: glycine/glutamine/glutamic acid; +2: glycine/arginine/serine) but have no effect on EBNA1 translation or antigenic presentation levels. Moreover, substitution of the EBNA1 GAR-encoding mRNA sequence by the G-rich repetitive sequence of *Ateline herpesvirus* ORF73 (which encodes the GMP of the virus), showed that this domain is also able to inhibit EBNA1 translation and antigen presentation <sup>93</sup>.

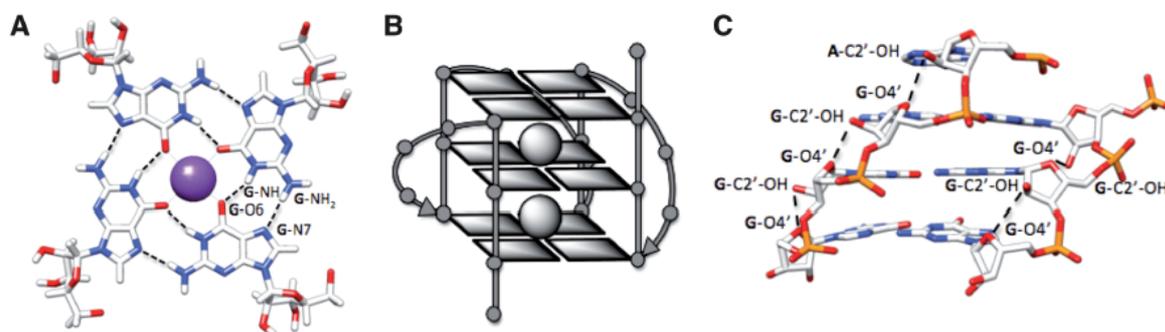
**Table 3-** Homologies between  $\gamma$ -herpesviruses GMPs. Table extracted from <sup>93</sup>.

| Gammaherpesviruses               | Viral maintenance protein homologues | Internal mRNA repeat size (bp) | Purine content of the mRNA repeats (%) | Identity of the nucleotide sequence repeat in EBNA1 with nucleotide repeats in other viral maintenance protein homologues (%) | Homology <sup>2</sup> of the protein sequence repeat in EBNA1 with repeats in other viral maintenance protein homologues (%) |
|----------------------------------|--------------------------------------|--------------------------------|--|---|--|
| <i>Lymphocryptoviruses</i>       |                                      |                                |  |   |  |
| <i>Human herpesvirus 4</i>       | EBNA1                                | 711                            | 88.1                                   | 100   | 100  |
| <i>Macacine herpesvirus 4</i>    | rhEBNA1                              | 141                            | 78.0                                   | 70.2  | 46.8   |
| <i>Papiine herpesvirus 1</i>     | baEBNA1                              | 49                             | 80.9                                   | 75.6  | 55   |
| <i>Rhadinoviruses</i>            |                                      |                                |  |   |  |
| <i>Human herpesvirus 8</i>       | LANA1 CR1 <sup>1</sup>               | 1818                           | 76.2                                   | 52  | 0.3  |
|                                  | CR2                                  |                                |  | 50  | 0.3  |
|                                  | CR3                                  |                                |  | 72  | 0.4  |
| <i>Macacine herpesvirus 5</i>    | ORF73                                | 1554                           | 66.5                                   | 57.1  | 2.1  |
| <i>Saimirine herpesvirus 2</i>   | ORF73                                | 546                            | 85.3                                   | 58.5  | 17.6   |
| <i>Macaviruses</i>               |                                      |                                |  |   |  |
| <i>Alcelaphine herpesvirus 1</i> | ORF73 CR1                            | 2061                           | 96.6                                   | 56.6  | 29.5   |
|                                  | CR2                                  |                                |  | 62.6  | 0  |
|                                  | CR3                                  |                                |  | 74  | 50   |
| <i>Ovine herpesvirus 2</i>       | ORF73 CR1                            | 984                            | 85.7                                   | 65.8  | 40.8   |
|                                  | CR2                                  |                                |  | 64  | 0  |
|                                  | CR3                                  |                                |  | 68.4  | 44   |

Finally, and further supporting a crucial role for the GAR-encoding mRNA sequence, the group of J. Tellam demonstrated *in vitro* that the region of the mRNA encoding the GAR domain forms unusual secondary structures known as G-quadruplexes (G4) <sup>99</sup>. Using circular dichroism (CD) spectroscopy they showed that a G-rich sequence of 18 nucleotides that occurs 13 times all over the GAR sequence of EBNA1 is able to form stable G4s. They proposed that this particular mRNA structure would cause ribosome stalling thereby leading to translation inhibition.

### 2.3.2.1 G4 organization

G-quadruplexes are non-canonical intra or intermolecular secondary structures of nucleic acids that form within G-rich DNA or RNA sequences. They are composed of and stabilized by the stacked of guanine tetrads which are assembled in a planar arrangement by Hoogsteen hydrogen bonding (**Figure 9**). The stability of the structure relies on the presence of a cation, in general  $K^+$  or  $Na^+$ , which is located inside or between the tetrads and coordinates the structure <sup>100</sup>. G4 can adopt several topologies depending on the orientation of the DNA or RNA strands; they can be parallel, antiparallel or hybrid. The canonical G4 motif is  $G_{\geq 3}N_xG_{\geq 3}N_xG_{\geq 3}N_xG_{\geq 3}$  in which the planar quartets are connected by loops containing 1-7 nucleotides (N). Putative G4-forming sequences are frequently present within the human genome which would contain 360.000 of such motifs according to bioinformatics predictions <sup>100</sup>. The yeast *Saccharomyces cerevisiae* would contain only twenty seven putative G4-forming sequences which may reflect the increase significance of these structures during evolution <sup>101</sup>. Some regions of the human genome like the telomeres or the ribosomal DNA (rDNA) are enriched in G4-forming sequences, contrary to coding portions of the genome.



**Figure 9-** Schematic representation of G-quadruplex structures. Figure extracted from <sup>102</sup>.

A) G-quartet arrangement, B) G-quadruplex secondary structure and C) the intermolecular hydrogen bonding between RNA G-quartet forming residues.

### 2.3.2.2 G4 functions in cell biology

G4 may constitute key regulatory elements as they can affect several steps in gene regulation as DNA replication, transcription, mRNA stability and translation. In addition, G4 can also impact mRNA localization.

#### a) *G4 in DNA*

When present inside the DNA, G4 can modify chromatin condensation and also gene transcription. Bioinformatics studies have shown that at least one G4 motif is present in almost 40% of human promoters and located 1000 nt upstream of the TSS (transcription starting site) sequence, which highlights G4 potential in transcription regulation. Depending on their position, G4 can either inhibit transcription if they are in the template strand, or enhance transcription when they are present in the non-template strand. G4 are often present in the promoters of human oncogenes while their frequency in tumor suppressor genes and housekeeping genes is dramatically lower<sup>103</sup>. Because of this, G4 present in promoters of human oncogenes are viewed as therapeutic targets as stabilization of these motifs may represent an attractive new anticancer strategy. In bacteria and yeast *Saccharomyces cerevisiae* there is also an enrichment in putative G4 motifs present within promoters which also reflects their importance during evolution<sup>104, 105</sup>.

The best studied G4 among those present in human promoters are those present within c-MYC promoter. c-MYC is a transcription factor which expression favors proliferation and as such is often overexpressed in human cancers. A particular nuclease hypersensitive element III (NHE-III) sequence present downstream the c-MYC promoter controls more than 80% of its transcription. In 2002 the team of L.H Hurley showed by footprinting experiments that the NHE-III element forms G4 structures which are involved in c-MYC transcription inhibition. The disruption of one nucleotide critically involved in G4 formation increased c-MYC transcription and inversely, TMPyP4 (a known G4 ligand) treatment in lymphoma cell lines showed antitumor activity due to G4 stabilization and thus transcription repression of c-MYC<sup>106</sup>.

### b) G4 in RNA

During the past few years several studies have shown the importance of G4 inside RNAs. Due to the fact that RNA does not contain a complementary strand, G4 formed inside RNA are usually more stable than their DNA counterparts<sup>107</sup>.

Most of G4 present in RNA are located in the 5' or 3'-UTR where they can affect translation or polyadenylation, but they can also have an effect on RNA splicing and mRNA turnover. Of note, potential G4 forming sequences within RNA are also present in telomeres where they can impact telomere maintenance.

Due to the ability of G4 to inhibit translation of its own (EBNA1) mRNA, the next section will focus on evidences indicating how G4 present inside mRNAs can affect translation.

### c) G4 formed in mRNAs can affect the translation rate.

Bioinformatics studies have revealed the presence of G4 forming sequences in at least 8% of 5'-UTR of human genes<sup>108</sup>, which highlights their importance as regulatory elements.

The 5'-UTR is a key element that ensures cap-dependent translation, as the small unit of the ribosome (40S) binds first to this sequence and then starts to scan the mRNA towards the 3'-end until it finds the first initiation (AUG) codon where the association with the large subunit of the ribosome (60S) takes place.

Thus, the presence of G4 in the 5'-UTR of RNA can affect translation initiation by preventing the binding of the initiation complex. G4 can also disturb the scanning of the mRNA by the 40S and affect the recruitment of translation initiation factors (eIFs). Hence, these secondary structures may interfere with cap-dependent translation. As a consequence, they can favor the cap-independent translation by internal ribosomal entry sites (IRES) which are present in many mRNAs<sup>109</sup>. One outstanding example concerns the vascular endothelial growth factor (VEGF) for which a G4 motif present in its 5'-UTR is essential to ensure IRES-mediated translation, which is itself critical under hypoxia<sup>110</sup>.

The first RNA G4 identified that can repress translation was found in the oncogene *NRAS*. As *KRAS*, its activation induces cell proliferation, differentiation and apoptosis. Translation control of *NRAS* occurs via the stabilization or destabilization of a G4 structure present in the 5'-UTR of its mRNA.

Mutations that cause the loss of the G4 motif increase *NRAS* expression and thus cell proliferation as in cancer cells. This makes *NRAS* G4 a therapeutic target as G4 ligands able to stabilize G4 will provoke a decrease in *NRAS* translation and thus apoptosis of cancer cells<sup>111</sup>. The author's also shown that only the G4 motifs of *NRAS* present near the 5'-UTR (as in wild type conditions) is able to block the formation of the initiation complex whereas the delocalization of the motif (from +14 to +120) completely abolishes the translation inhibition. This observation reflects the importance of G4 position inside the mRNA in order to impede translation.

When found in the 3'-UTR, G4 can cause translation repression. This is the case for the proto-oncogene *PIM1* which encodes a serine/threonine kinase involved in cell growth, transcriptional activation and apoptosis<sup>112</sup>.

#### 2.3.2.3 G4 binding proteins and their role in G4 stability.

Several proteins have been identified for their ability to bind G4 structures formed in DNA or RNA molecules. These G4 binding proteins can be classified in two groups: G4 binding proteins that destabilize G4 upon binding, and G4 binding proteins that stabilize G4 upon binding. These G4-binding proteins can be recruited to G4 in response to various cellular stresses or cell cycle changes for example.

##### *a) G4 binding proteins that destabilize G4 upon binding*

In this category 3 main families of proteins can be found: heterogeneous nuclear ribonucleoproteins (hnRNPs), telomere-related G4 binding proteins (shelterin complex) and the transcription factor MAZ.

The heterogeneous nuclear ribonucleoprotein (hnRNPs) are DNA/RNA binding proteins involved in several steps of the nucleic acid metabolism like alternative splicing and translation and also in telomere protection. This is the case of the hnRNP D which is able to bind specifically to the 3' end of the human telomeric sequences and unfold a G4 motif that is able to inhibit telomerase activity<sup>113</sup>. This is also the case for hnRNP A which is also able to unwind the telomeric G4 thereby enhancing telomerase activity. This was demonstrated *in vitro* showing that depletion of hnRNP A from cells

dramatically decreases telomerase activity, and that this can be reverted by addition of recombinant hnRNP A1<sup>114</sup>.

hnRNP A1 is also able to affect transcription of various genes. This is the case of *KRAS* for which hnRNP A1 binds and destabilizes the G4 motif present in the NHE element upstream of the *KRAS* promoter thereby activating the transcription of this oncogene<sup>115</sup>.

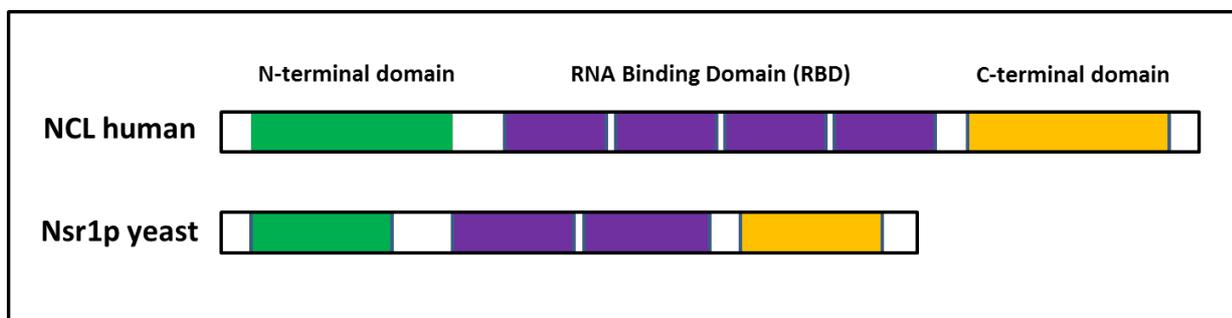
In addition, several proteins from the Shelterin complex have been shown to bind to telomeric G4 sequences. The shelterin complex is formed by 6 proteins (POT1, TRF1, TRF2, RAP1 and TPP1 and TPP2) which are involved in telomere maintenance and genome stability. The group of S. Myong demonstrated in 2012 the mechanism by which POT1, in coordination with TPP1, regulates both G4 folding and unfolding thereby ensuring telomerase processivity<sup>116</sup>. POT1 binds to G4 formed within telomere repeats in a 3'-5' direction and starts G4 unfolding by a 2 steps process, after which TPP1 is recruited and the complex between these two proteins causes a sliding that leads to a switch from a folded to an unfolded state, which itself affects telomerase processivity. Another well characterized telomeric G4 binding protein is the replication protein A (RPA). It has been demonstrated that RPA is able to regulate telomerase function during cell cycle by causing the unfolding of G4 formed within telomeric DNA, thus allowing telomerase binding<sup>117</sup>.

Finally the zinc-finger transcription factor MAZ is able to bind and destabilize the G4 sequences present in the *HRAS* promoter thus favoring the formation of a DNA duplex instead of quadruplex, thereby allowing transcription initiation<sup>118</sup>.

*b) G4 binding proteins that stabilize G4 upon binding:*

In this category, the most studied and well-known G4 binding protein nucleolin (NCL) will be described. Nucleolin was first described in 1973 by the group of H. Busch that called it C23 according to its position in two dimensional gel electrophoresis<sup>119</sup>. NCL is a multifunctional DNA and RNA binding protein widely conserved among eukaryotes. It is mainly localized in the nucleolus and in good agreement with this particular localization it has been involved in RNA metabolism, in particular, ribosomal RNA (rRNA) maturation. Most of its protein domains and functions are conserved from yeast to human. Human NCL is a 713 amino acids protein with a predicted molecular weight of 77 kDa, although it migrates as a 100-110 kDa protein. This discrepancy is attributed to the

numerous post-translational modifications (phosphorylation, methylation, ADP-rybosilation, etc.) of the acidic N-terminal domain of the protein <sup>120</sup>. In addition to its acidic N-terminal domain, NCL contains four central RNA-binding domains (RBD) involved in its interaction with mRNAs and pre-mRNAs. Of note, while human NCL contains 4 RBD, yeast *Saccharomyces cerevisiae* NCL only possesses 2 RBD (**Figure 10**). These RBD have less sequence homology between them than with the equivalent RBDs from other species <sup>120</sup>. NCL is a RNA-binding protein that binds to its target RNA on a consensus motif (U/GCCCGA) that forms part of a short stem-loop structure of at least 18 nucleotides <sup>121</sup>. Finally the C-terminal domain of NCL is a glycine-arginine rich (RGG or GAR) domain involved in NCL interaction with ribosomal proteins. Of note, this GAR domain should not be mixed up with the GAR domain of EBNA1. As it is also called RGG domain, we will use this nomenclature in order to avoid confusion. The RGG domain has been implicated in the assembly of pre-ribosomal particles and also controls NCL nucleolar localization. Interestingly, this RGG domain is essential for an efficient binding of the RBDs to their target RNA as it is able to modify the binding properties of the RBDs <sup>122</sup>. It was also demonstrated that the RGG domain can bind to mRNA and affect their translation.



**Figure 10-** Human and yeast nucleolin protein organization.

In green is represented the acidic N-terminal domain, in violet all RNA-binding domain (RBD) and in orange the C-terminal RGG domain.

As stated above, more than 90% of NCL is located in the nucleolar compartment. However it has been shown to be also present in the nucleocytoplasmic fraction and at the cell surface. Within the nucleolus, NCL is mainly present inside the Dense Fibrillar Compartment (DFC), much less in the Granular Compartment (GC) and sometimes it is also present in the Fibrillar Center (FC). Since NCL has pivotal roles in the rRNA synthesis and ribosome biogenesis it has been proposed that it's

shuttling between nucleus and cytoplasm may be involved in the transport of ribosomal components. Thereby this change of localization plays an important role in ribosomal biogenesis.

NCL has been reported to interact with several DNA and RNA sequences. When binding to DNA it can enhance or repress transcription. For example, NCL has been shown to bind to the NHEIII element present upstream of the promoter of c-MYC gene and to inhibit its transcription by stabilizing its G4-forming sequences <sup>123</sup>. NCL can also alter the chromatin condensation thereby affecting DNA replication. It is able to bind RNA sequences in particular in the 5'-UTR of some RNAs which may affect their translation. NCL has also been shown to bind to the 3'-UTR of some mRNAs which may affect their stability. Finally, NCL can also bind to the coding sequence of some mRNAs thereby affecting their translation.

#### 2.3.2.4 Human pathogens using G4 structures in order to evade the immune system recognition.

G4 structures have been widely described from yeast to human. In addition, in the past few years it has been shown that they can also be formed in the genome or transcripts of several human pathogens, and that they may help those pathogens to evade the immune system (**Table 4**). The antigenic variation (Av) is the process by which pathogens express different versions of a surface antigen in order to evade the immune system. They possess several variants of this antigen but they express only one of them at a time. The switch between the different variants can be due to site-specific recombination, epigenetic changes, or homologous recombination, the latter being the most common <sup>124</sup>. In addition, G4 structures can also be implicated in the Av of pathogens. This is the case for *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Both of them express three major antigens that are involved in bacterial survival. The pilin proteins are essential for establishing the infection as they are implicated in cellular adherence and bacterial aggregation <sup>125</sup>. *pilE* is the major component of the pilus and Av occurs by recombination of this locus with several silent variants called *pilS*. The Av of *N. gonorrhoeae* is controlled by a DNA G4 motif located upstream the *pilE* region which controls its transcription rate. The treatment with G4 ligands that stabilize G4 formation inhibits the frequency of Av by decreasing the recombination of *pilE* with *pilS* loci <sup>126</sup>. Furthermore, *pilE* G4 motif recruits *N. gonorrhoeae* RecA helicase in order to unwind the structure and thus facilitate the recombination, thereby allowing the Av <sup>127</sup>.

G4 forming sequences have also been identified in eukaryotic pathogens like *Plasmodium falciparum*, the parasite causing malaria in humans. *P. falciparum* is able to enter into erythrocytes and to provoke their attachment to microvascular endothelial cells leading to vascular obstruction. The only protein involved in this process is the *P. falciparum* erythrocyte membrane protein one (PfEMP1) which is encoded by at least 50 different *var* genes. Each parasite expresses only one *var* gene at a time and can switch to other variants to generate the Av. As in *N. gonorrhoeae*, evidence suggests that some DNA G4 motifs located upstream of the *var* promoter can control *var* transcription and thus Av<sup>128</sup>.

**Table 4-** G-quadruplex structures found in pathogens. Table adapted from<sup>129</sup>.

|                              | Pathogen               | G4 localization  | G4 function                              | Virulence phenotype |
|------------------------------|------------------------|--|--|---------------------|
| <b>Viruses</b>               | HPV                    | LTR, L2, E1, E4 (Tlučková <i>et al</i> 2013 <sup>130</sup> ) | DNA transcription regulation             | VL                  |
|                              | EBV                    | EBNA1 (Murat <i>et al</i> 2014 <sup>99</sup> )               | mRNA translation, antigenic presentation | VL                  |
|                              | HIV-1                  | LTR (Perrone <i>et al</i> 2013 <sup>131</sup> )              | DNA/RNA transcription                    | VL/AD               |
| <b>Prokaryotic pathogens</b> | <i>N. meningitidis</i> | <i>pilE</i> (Wörmann <i>et al</i> 2014 <sup>132</sup> )      | DNA breakpoints                          | Av                  |
|                              | <i>N. gonorrhoeae</i>  | <i>pilE</i> (Kuryavyi <i>et al</i> 2012 <sup>127</sup> )     | DNA breakpoints                          | Av                  |
| <b>Eukaryotic pathogens</b>  | <i>P. falciparum</i>   | Var (Smargiasso <i>et al</i> 2009 <sup>128</sup> )           | DNA transcription regulation             | Av / AD             |

G4 motifs are also present in some human viruses and are implicated in viral replication and immune evasion. This is the case for the human immunodeficiency virus type 1 (HIV-1), human papillomavirus (HPV) and EBV. In the case of HIV-1, its long terminal repeats (LTR) control transcription of several genes and also of the full-length viral genome of the integrated provirus. The LTR promoter contains two intramolecular G4 sequences whose formation inhibits the transcription initiation. R. Perrone *et al* demonstrated that a treatment with G4 stabilizing ligands inhibits LTR-mediated transcription thus leading to a decrease in HIV-1 infectivity. Conversely, mutations that abolish G4 formation increase promoter activity<sup>131</sup>. Other G4 forming sequences have been reported in the coding sequence of the *nef* gene. The Nef protein is expressed during the early phase of HIV-1 life cycle and is essential for viral replication and infectivity. The stabilization of the G4 motifs present within *nef* coding sequence inhibits its expression and thus decreases HIV-1 infectivity. These observations highlight the importance of G4 structures in viral infection. For these reasons, many efforts were made in order

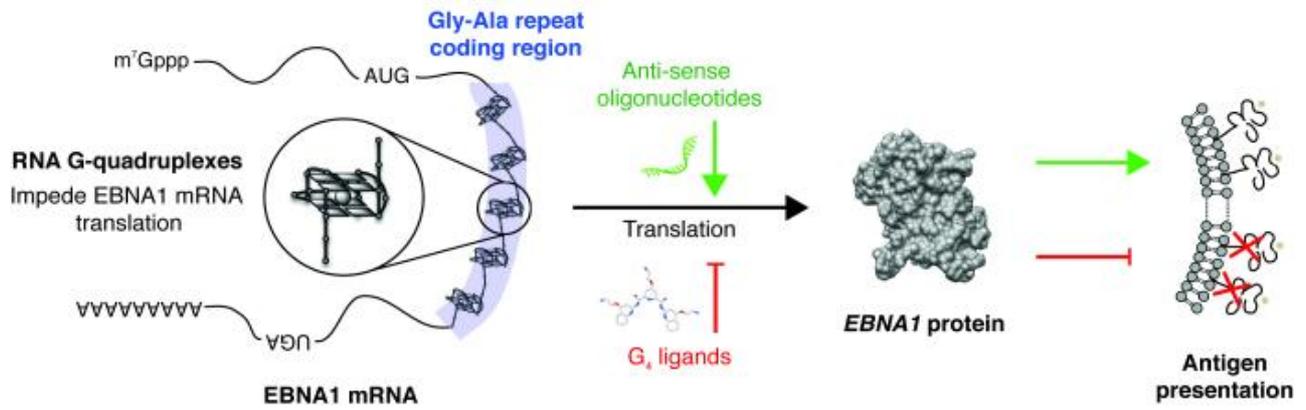
to identify cellular or viral proteins that could interact with these G4 and affect their stability. In this context, E. Tosoni *et al* used a combination of electromobility assays (EMSA) and mass spectroscopy to identify cellular and viral proteins interacting with G4 within LTR sequences <sup>133</sup>. They found that Nucleolin is able to specifically bind and stabilize G4 present inside the LTR and that this interaction causes a decrease in viral transcription. This interaction could be important for the first switch of the virus life cycle to the latency state as proteins able to attenuate transcription are important in order to consolidate latency. Nevertheless, it has been demonstrated that cell surface NCL is essential to ensure HIV-1 attachment and entry inside the cells since a treatment with AS1411, a G4 oligonucleotide which binds and traps the fraction of NCL localized at the cell surface, leads to a decrease in HIV-1 entry into the cells <sup>134</sup>.

Regarding HPV, several G4 forming sequences have been identified in its genome notably inside the LTR, the minor capsid component L2 and the early genes E1 and E4 <sup>130</sup> suggesting that these G4 may have an effect on HPV replication and transcription.

Finally, regarding EBV, G4 forming sequences have been identified in the EBNA1-encoding mRNA. P. Murat *et al* demonstrated that the mRNA sequence of EBNA1 encoding the central GAR domain may form several G4. *In vitro* studies have shown that these G4 present inside the GAR domain coding sequence of EBNA1 are able to inhibit translation, thereby leading to immune evasion during the latent phase <sup>99</sup>. P. Murat *et al* proposed that EBNA1 G4 may cause ribosome stalling and therefore inhibit translation. The treatment with pyridostatin (PDS), a G4 ligand known to stabilize G4, causes a decrease in EBNA1 inhibition of translation whereas the treatment with antisense oligonucleotides that destabilize G4 structure enhances EBNA1 translation and promotes T CD8<sup>+</sup> recognition (**Figure 11**).

These experiments showed that targeting the GAR domain with G4 ligands may interfere with EBNA1 translation inhibition and would thus modify the rate by which EBNA1-derived antigenic peptides are presented by MHC class I.

As of today, both GAR polypeptide-based and G4-based hypotheses are still topical and the cellular factors involved in GAR-based immune evasion are still unknown.



**Figure 11-** Model representing the mechanism of action by which the G4 motifs present inside the GAR-encoding mRNA of EBNA1 allow immune evasion of the EBV-infected cells in latent phase. Figure extracted from <sup>99</sup>.

The G4 present in GAR mRNA inhibit translation of EBNA1. The treatment with G4 ligands, that stabilize G4 structures, decreases EBNA1 protein level and thus antigen presentation at the cell surface. In contrast anti-sense oligonucleotides that disrupt the compact G4 motifs enhance translation and antigenic presentation.

## CHAPTER III: GAR-based immune evasion is a therapeutic target for cancer: need of a model.

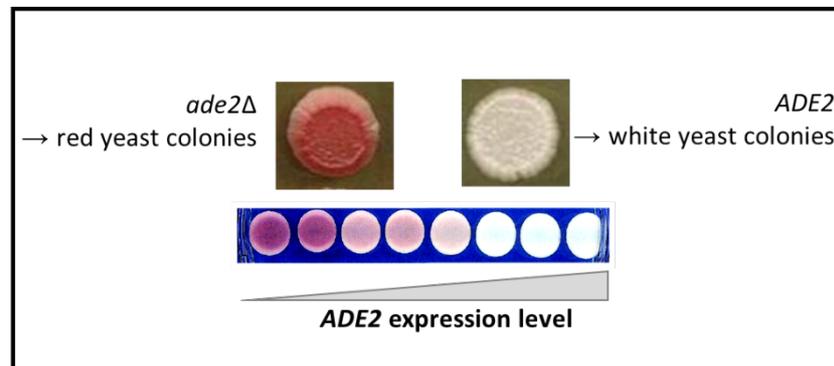
The efficient T cell response against EBV-infected cells in which the GAR domain of EBNA1 has been deleted demonstrates the importance of this domain in the viral strategy to evade antigen presentation <sup>91</sup>.

The mechanism by which the GAR domain self-limits the translation of its own mRNA *in cis* clearly represents a relevant therapeutic target to treat EBV-related cancers. Indeed, most tumoral cells of EBV-related cancers are EBV-infected, contrary to the vast majority of non-tumoral cells. Hence, interfering with the GAR-based inhibition of translation should unveil tumoral cells to T CD8<sup>+</sup> raised against EBNA1 that exist in every EBV-infected individual. For this purpose, a relevant cell-based assay was required.

In order to create a GAR-based model, yeast looks very appropriate. Indeed, the versatile genetic flexibility of the budding yeast *Saccharomyces cerevisiae* and the high degree of conservation between yeast and mammalian cellular processes have made *S.cerevisiae* an invaluable tool for modeling human diseases <sup>135-137</sup>, as well as for identifying and characterizing cellular pathways involved in these disorders and thereby new therapeutic targets <sup>138, 139</sup>. Moreover, EBNA1 has been expressed in yeast to study its crucial role in EBV episome maintenance <sup>140-142</sup>. These studies showed that a functional EBNA1 protein can be expressed in yeast and indirectly suggested that the GAR domain might affect mRNA translation in this unicellular organism. Therefore, we hypothesized that a broad approach using yeast would be suitable to identify modulators (either drugs or genes) that could interfere with the GAR-mediated inhibition of translation whatever the exact mechanism of this regulation process.

We thus set up a yeast-based assay that recapitulates the effect of the GAR domain on translation regulation <sup>143</sup>. This yeast-based assay consists in the use of a *Saccharomyces cerevisiae* strain in which the yeast *ADE2* gene encoding the phosphoribosylaminoimidazole carboxylase (AIR), involved in the adenine synthesis pathway, was deleted (*ade2Δ* strain). As a consequence of this deletion, colonies of *ade2Δ* cells present a red phenotype due to the accumulation of AIR which becomes red when oxidized by active respiration (**Figure 12**). This *ade2Δ* strain was used to express an *ADE2* construct which restores the normal adenine synthesis thus resulting in a white phenotype of yeast cells as well as several *GAR-ADE2* fusions which lead to pink to red phenotypes due to the length-dependent

translation inhibition exerted by the GAR domain<sup>143</sup> (**Figure 13A**). Any compound or gene that would act on the translation inhibition exerted by the GAR domain would cause a modification of the GAR-*ADE2* expression level and give rise to a whiter or a redder phenotype (**Figure 13B**).



**Figure 12-** The yeast reporter system *ADE2*.

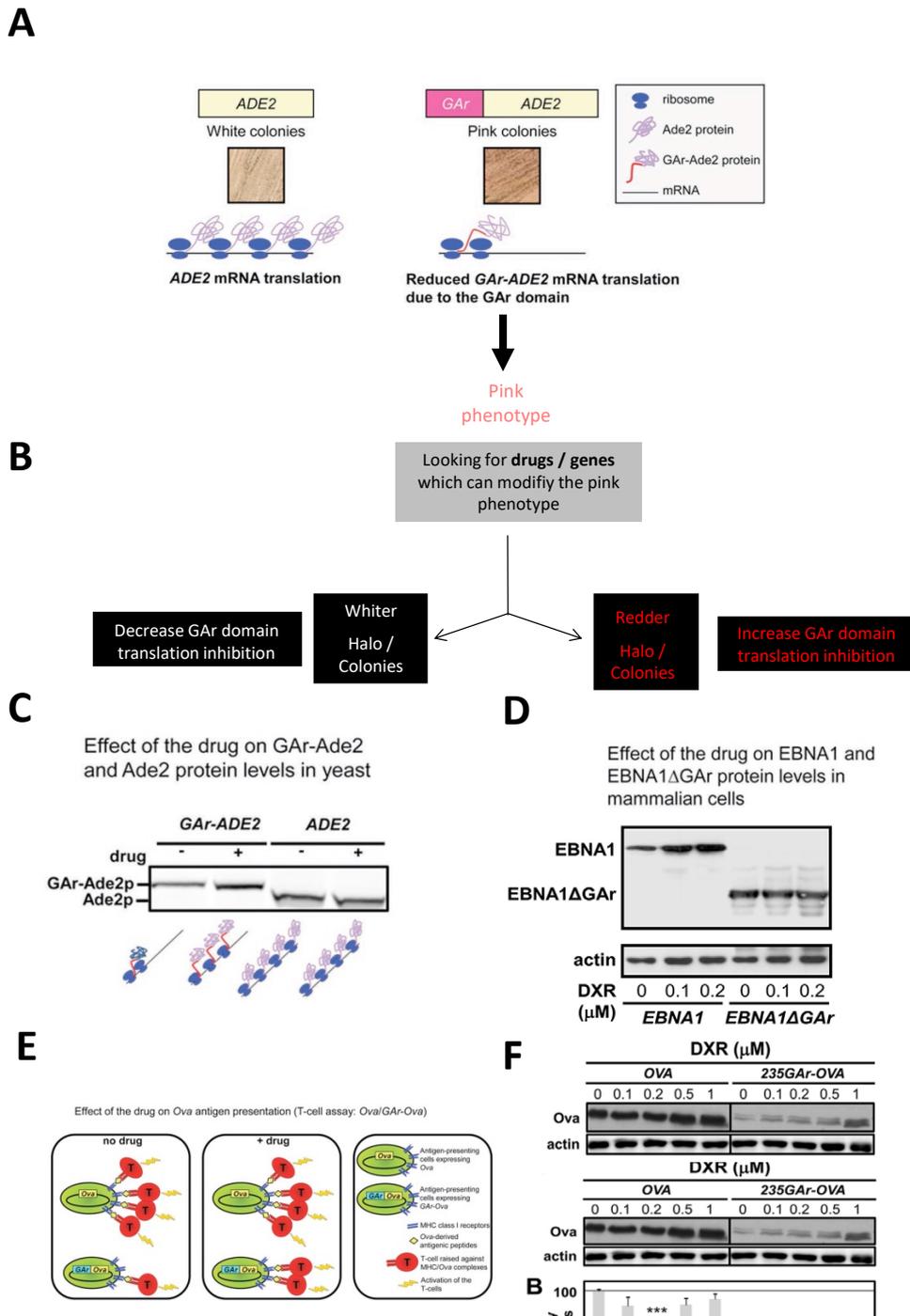
Yeast colonies from a *ade2Δ* strain present a redder phenotype due to the accumulation and oxidation of AIR (phosphoribosylaminoimidazole) which is an intermediate metabolite of the adenine synthesis. In contrast, the full expression of *ADE2* causes a yeast white phenotype. The color of the yeast colonies is proportionate to *ADE2* expression level.

For this purpose, we selected the 43GAR-*ADE2* yeast strain that has a pink phenotype which enables the identification of compounds or genes able to affect GAR inhibition of translation. The pink phenotype of this strain should allow to identify compounds (or genes) that cause a redder phenotype, which means that there is an increase in GAR domain inhibition of translation, or a whiter phenotype that signifies an inhibition of GAR effect (**Figure 13B**). Both phenotypes are equally interesting since a decrease in EBNA1 expression level would be deleterious for the virus genome replication and maintenance due to EBNA1 essential functions; and an increase of EBNA1 expression level would cause the recognition of virus-infected cells by the host immune system.

We used this yeast model to perform a drug screening of FDA-approved compounds and isolated doxorubicin (DXR) which causes a change of color of 43GAR-*ADE2* strain from pink to white (**Figure 13B**). This results were confirmed by western blot showing that DXR treatment increases the expression of 43GAR-*ADE2*p without affecting *ADE2*p level. This effect was due to an increase in protein synthesis (**Figure 13C**) without changing mRNAs levels<sup>143</sup>. After having identified a candidate

drug, results were confirmed in mammalian cells by expressing two different types of constructs: EBNA1 / EBNA1ΔGAR and 235GAR-OVA / OVA constructs which are essential in order to further test the capacity of DXR to alter the antigenic presentation (**Figure 13D**). After validation of DXR activity in mammalian cells the last step was to test if indeed it is able to affect antigen presentation. For that we evaluated the capacity of T CD8<sup>+</sup> cells against the SL8 antigenic peptide of OVA to recognize 235GAR-OVA after the treatment with DXR. The treatment with DXR causes a dose-dependent increase in 235GAR-OVA antigen presentation without affecting OVA antigen presentation (**Figure 13E-F**).

Thus DXR, the drug we isolated thanks to this model, affects the GAR domain-dependent inhibition of translation, both in yeast and in human cells, and also the antigenic presentation<sup>143</sup>. This means that the mechanism involved in the GAR-mediated regulation of translation is conserved from yeast to human. It also validates this yeast-based assay for isolating compounds or cellular factors that could interfere with the EBNA1-dependent immune evasion of EBV.



**Figure 13-** Yeast-based assay for EBNA1-based immune evasion. Figure adapted from <sup>2, 3</sup>.

A. GAR domain of EBNA1 responsible for EBNA1 translation inhibition *in cis* was fused to the reporter gene *ADE2*. The expression of Ade2p gives white colonies whereas the inhibition of its translation by GAR gives pink colonies (*GAR-ADE2* construct). This pink phenotype (B) was used to isolate compounds able to affect this GAR translation inhibition. Drugs able to suppress GAR effect will give white colonies which can be easily monitored in yeast (C) or in human cells (D). Finally, drug effect on antigen presentation is determined using a T cell assay (E-F).

## Aim of the PhD

Viruses have evolved with their hosts all over the animal kingdom. Some of them, like the  $\gamma$ -herpesviruses, are able to establish lifelong infections of their hosts without being detected by the immune system. Their camouflage is based on the control of the expression of a unique viral protein, named GMP (genome maintenance protein), which has two essential missions: first, it ensures the replication of the viral genome during the S phase of the cell cycle; second it acts as a bridge to tether the viral episome to the host chromosomes. One of the most studied GMP is the GMP of the Epstein-Barr virus (EBV): EBNA1. Like all the GMP, EBNA1 is highly antigenic and T cells towards EBNA1 exist in all EBV-infected individuals. In order to hide from the host immune system, EBNA1 is able to control its own mRNA translation and thus the presentation of EBNA1-derived antigenic peptides, a process controlled by its central GAR domain.

Due to the antigenicity of EBNA1 and the mechanism that tightly regulates its own mRNA translation, the EBNA1 protein constitutes a good therapeutic target to treat EBV-associated cancers. Indeed, only few memory B cells are latently infected by EBV thus their destruction by the immune system should not be deleterious for the patients. However, the mechanism of translation inhibition of EBNA1 by its GAR domain is currently undeciphered and the cellular factors involved in this regulation have not been identified yet.

The aim of my PhD was to identify cellular factors that are involved in this GAR domain effect. These cellular factors are potential therapeutic targets that will help to interfere with the GAR-based translation regulation. To identify such cellular factors, we took advantage of the yeast model which recapitulates the effect of GAR in the inhibition of translation (**Figure 13**), to perform a genetic screening to identify genes which overexpression could affect GAR self-inhibition. Thanks to this screening we identified Nucleolin (NCL). We first demonstrated the involvement of NCL in the GAR-based translation inhibition and then we went deeper into the mechanism by which NCL is involved in the GAR domain inhibition of translation.



# Results section organization

The results section is organized in three chapters. The first chapter comprises the discovery of Nucleolin (NCL) as the first GAR domain mRNA cellular partner, and also how this interaction can constitute a therapeutic target to treat EBV-associated cancers. These results were the subject of a first publication which has been submitted to Nature Communications in October 2016.

The second chapter describes more deeply the mechanism involved in this NCL-GAR mRNA interaction: how, where and why it takes place. These results will be part of a second manuscript which is currently in preparation.

Finally, the third chapter corresponds to a review article that was published in 2015 in Biotechnology journal, which described how the yeast *Saccharomyces cerevisiae* happened to be a good model to study the different steps in the Epstein-Barr virus life cycle.

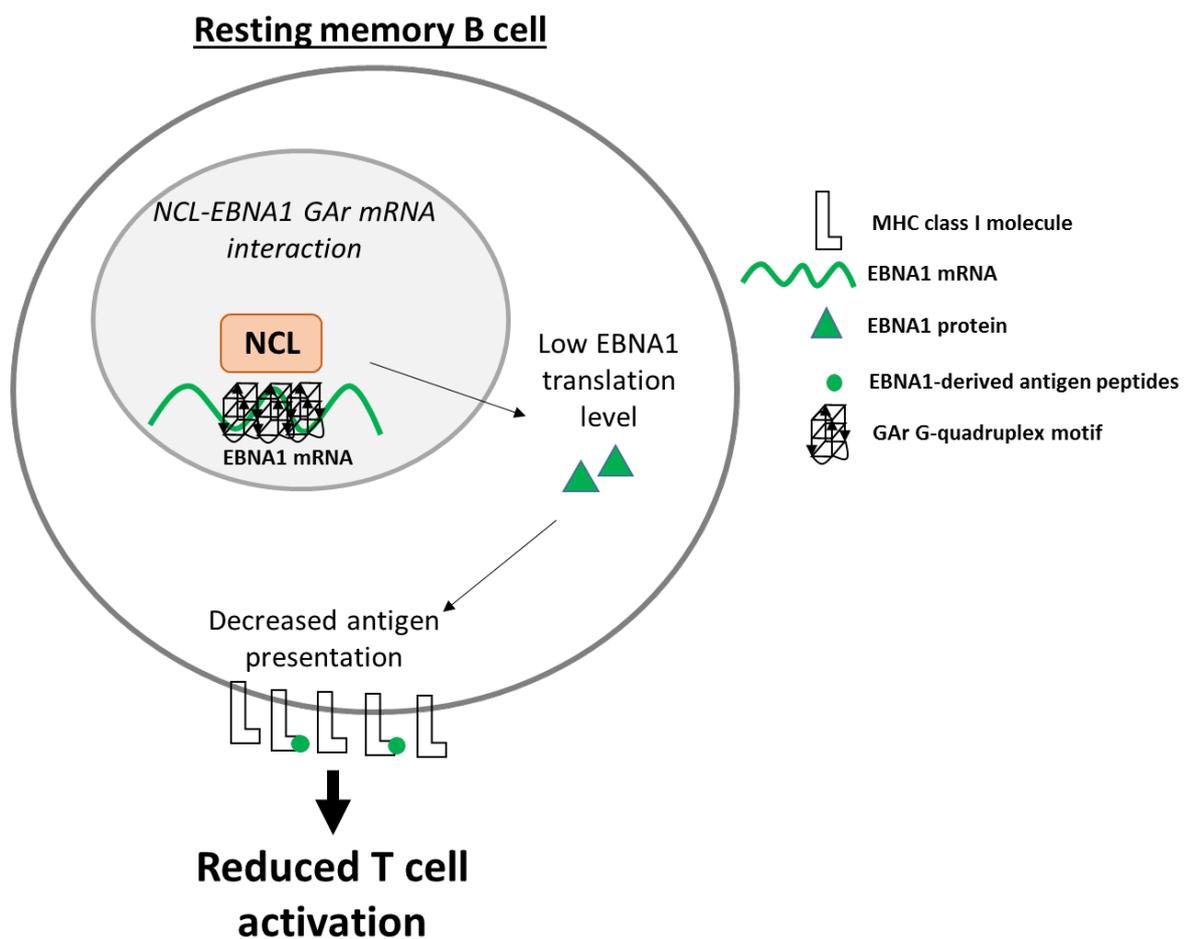


# Results

## Chapter 1:

Identification of NCL as the first cellular factor involved in the GAR domain regulation of translation

- **Graphical abstract:**



- ***Manuscript presentation:***

During this thesis, we studied the mechanism of the GAR domain-based inhibition of translation in order to identify cellular factors that are able to enhance or inhibit this translation regulation. Thanks to the genetic screening we performed in the yeast model that recapitulates the GAR domain-based regulation of translation, we were able to identify several candidate genes which overexpression alters the GAR-dependent translation inhibition. We focus our interest on *NSR1* and its human homolog Nucleolin (NCL). So why did we get interested in NCL? First, it happens that NCL is a well characterized DNA/RNA binding protein which has been implicated in many cellular functions such as ribosome biogenesis, rRNA transcription, cell cycle control, apoptosis, transcription and translation regulation<sup>120</sup>. Second, many evidences showed that NCL has the ability to bind to G4 structures present on promoters (like c-Myc) and through this interaction NCL can modify genes transcription rate<sup>123, 144</sup>. Similarly, NCL can bind to G4 on the 5'-UTR or 3'-UTR of mRNAs and affect translation or mRNA stability. Moreover NCL has recently been shown to interact with the N-terminal domain of EBNA1 protein and enhance EBNA1-mediated OriP transcription.

The fact that there was already a demonstration that NCL can affect one of the two functions of EBV GMP, that the GAR domain mRNA is able to form G4 motif, and that NCL is a G4 binding protein indicate that NCL constitutes an excellent candidate and led us to focus primarily on this cellular protein and to study its effect on the GAR domain inhibition of translation.

In our first manuscript we demonstrated that NCL overexpression decreases GAR-OVA and endogenous EBNA1 protein level, and inversely that NCL downregulation enhances GAR-OVA and EBNA1 translation. Increasing GAR-OVA protein levels consequently enhances the presentation of OVA-derived antigenic peptides at the surface of APC and also the recognition by T CD8<sup>+</sup> cells directed against OVA. Furthermore, we showed for the first time that NCL interacts with GAR mRNA through a direct interaction with the G4 motifs present inside the GAR-encoding sequence, and also that this interaction takes place in the nucleus. Finally, we demonstrated that targeting this interaction, for example by using small G4 interacting compounds like PhenDC3, interferes with the GAR domain translation inhibition thus demonstrating that NCL-EBNA1 mRNA interaction represents a novel and relevant therapeutic target to treat EBV-associated cancers.

These data led to the redaction of a publication that has been submitted to Nature Communications and which manuscript is placed below.

1 **Nucleolin is required for the Epstein-Barr virus-encoded EBNA1 immune**  
2 **evasion through binding to G-quadruplexes of EBNA1 mRNA**

3  
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5 Pierre Pochard<sup>3</sup>, Chrysoula Daskalogianni<sup>2</sup>, Corinne Guetta<sup>4</sup>, Christophe Jamin<sup>3</sup>, Marie-  
6 Paule Teulade-Fichou<sup>4</sup>, Robin Fåhraeus<sup>2</sup>, Cécile Voisset<sup>1\*</sup> and Marc Blondel<sup>1,5\*</sup>

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20  
21 **Running Title:** Nucleolin role in EBNA1 immune evasion

22  
23 **Keywords:** human (NCL) and yeast (NSR1) Nucleolin, Epstein-Barr virus (EBV), EBNA1, GAR domain,  
24 *Saccharomyces cerevisiae*, G-quadruplex, translation regulation, immune evasion, PhenDC3

25  
26 **Abbreviations:** EBV, Epstein-Barr virus; GMP, genome maintenance protein; GAR, glycine-alanine repeat;  
27 NCL, human Nucleolin; MHC, major histocompatibility complex; G4, G-quadruplex/es

28

29 **ABSTRACT**

30 **The oncogenic Epstein-Barr virus (EBV) evades the immune system but has an Achilles heel: its**  
31 **genome maintenance protein EBNA1. Indeed, EBNA1 is essential for viral genome**  
32 **maintenance but is highly antigenic. Hence, EBV evolved a system in which the glycine-alanine**  
33 **repeat (GAR) of EBNA1 self-limits the translation of its own mRNA at a minimal level to ensure**  
34 **its essential function thereby, at the same time, minimizing immune recognition. Here, we**  
35 **show that nucleolin (NCL) is required for this process *via* a direct interaction in the nucleus**  
36 **with G-quadruplexes formed in GAR-encoding mRNA sequence. Overexpression of NCL**  
37 **exacerbates GAR-based translation inhibition whereas its downregulation relieves the**  
38 **suppression of both translation and antigen presentation. Moreover, the G-quadruplex ligand**  
39 **PhenDC3 prevents NCL binding on EBNA1 mRNA and reverses GAR-mediated repression of**  
40 **translation and antigen presentation. Hence the NCL-EBNA1 mRNA interaction is a relevant**  
41 **therapeutic target to trigger an immune response against EBV-carrying cancers.**

42  
43 The Epstein-Barr virus (EBV) is the first oncogenic virus discovered in human<sup>1-3</sup> and has been  
44 linked to various cancers that include Burkitt and Hodgkin lymphomas and 10% of gastric  
45 cancers. Another example is the nasopharyngeal carcinoma which is particularly frequent among  
46 men in China and Tunisia. Like all the gammaherpesviruses, EBV evades the host immune system  
47 but has an Achilles heel: its genome maintenance protein (GMP) EBNA1<sup>4,5</sup>. Indeed, EBNA1 is  
48 essential for EBV genome replication and maintenance and as such expressed in all EBV-infected  
49 cells. On the other hand, EBNA1 is highly antigenic and CD8<sup>+</sup> T cells directed towards EBNA1  
50 epitopes exist in all infected individuals. Hence, EBV has evolved a mechanism to limit EBNA1  
51 production to the minimal level required for the viral genome replication and, at the same time,  
52 to minimize the production of EBNA1-derived antigenic peptides presented to the cytotoxic T  
53 cells through the MHC class I pathway<sup>4,6</sup>. The central glycine-alanine repeat (GAR) of EBNA1  
54 plays a critical role in this mechanism of immune evasion as it is able to self-inhibit the  
55 translation of its own mRNA *in cis*<sup>7,8</sup>. The high level of EBNA1 protein and the efficient T cell

56 response following the infection by an EBV strain encoding a truncated version of EBNA1 in  
57 which GAR has been deleted (EBNA1ΔGAR) demonstrates the critical role of GAR in EBNA1  
58 immune evasion<sup>7-10</sup>. Of note, a polymorphism in the length of GAR exists and, importantly, the  
59 effect of GAR is length-dependent as a longer domain displays a stronger inhibitory effect on  
60 both mRNA translation and antigen presentation<sup>11</sup>.

61 The GAR-encoding mRNA sequence is GC rich and forms predicted G-quadruplex (G4) structures  
62 that have been implicated in the regulation of EBNA1 synthesis *in vitro*<sup>12</sup>. G4 are particular  
63 secondary structures of nucleic acid formed by the stacking of G-quartets which correspond to a  
64 planar arrangement of 4 guanines connected by Hoogsteen hydrogen bonds. G4 structures  
65 within G-rich DNA or RNA sequences have been implicated in gene regulation where they can  
66 affect transcription, alternative splicing and translation<sup>13-17</sup>. G4 modes of action are still  
67 relatively unknown but cellular factors that can interact with these structures are emerging<sup>18-20</sup>.

68 Nucleolin (NCL) is a multifunctional DNA/RNA-binding protein widely conserved among  
69 eukaryotes. It is involved in RNA metabolism, in particular in rRNA maturation<sup>21</sup>. NCL binds to G-  
70 rich sequences in coding and non-coding regions of various mRNA, many of which encode  
71 cancer-related proteins, and enhance their translation<sup>22</sup>. In addition, NCL binds to some G4  
72 structures within DNAs and RNAs. For example, it has been recently shown that NCL binds to  
73 and stabilizes G4 structures formed within the LTR promoter of HIV, thereby silencing the  
74 provirus transcription<sup>23</sup>. NCL also affects the transcription of c-MYC by binding to and stabilizing  
75 G4 present in the promoter of this oncogene and that negatively regulate its activity<sup>17,24</sup>.

76 GAR-based EBNA1 immune evasion has been considered a relevant therapeutic target to treat  
77 EBV-related cancers as most tumor cells from EBV-related cancers are infected by EBV whereas,  
78 in healthy individuals, the latent infection by EBV is primarily restricted to a specific small pool of  
79 memory B cells. Hence, overcoming GAR-based self-inhibition of EBNA1 translation should unveil  
80 EBV-carrying tumor cells to cytotoxic T cells without having significant effect on the vast  
81 majority of healthy host cells. As the mechanisms of GAR-mediated mRNA translation

82 suppression *in cis* are not known, nor the cellular factors involved, we developed a yeast-based  
83 (*Saccharomyces cerevisiae*) assay that recapitulates all the aspects of the GAR-based inhibition of  
84 translation, including the GAR-length dependency<sup>25,26</sup>. This assay was successfully used to  
85 identify small molecular weight compounds that can stimulate EBNA1 expression both in yeast  
86 and mammalian cells and that relieve GAR-based limitation of antigen presentation<sup>26</sup>.  
87 Based on the same yeast assay, here we have performed a genetic screen to identify host cell  
88 genes involved in the GAR-mediated inhibition of translation. This way, we isolated the yeast  
89 *NSR1* gene which encodes the orthologue of human NCL and showed that NCL is critically  
90 involved in GAR-based limitation of translation and antigen presentation. We also found that  
91 NCL directly interacts in the nucleus with G4 formed in the GAR-encoding sequence of EBNA1  
92 mRNA. Finally, we show that this interaction is druggable and that the G4 ligand PhenDC3  
93 prevents NCL from binding to G4 formed in the GAR mRNA sequence and stimulates GAR-limited  
94 translation and antigen presentation.  
95 Hence, NCL represents the first host cell factor critically involved in EBNA1 immune evasion and  
96 the NCL-EBNA1 mRNA interaction appears to be a relevant therapeutic target to treat EBV-  
97 related cancers. Importantly, the results also show that G4 structures act as druggable protein  
98 binding modules.

99

## 100 RESULTS

### 101 Yeast nucleolin Nsr1p is required for GAR-based inhibition of protein expression in yeast and 102 human nucleolin NCL can functionally replace Nsr1p

103 The yeast assay used in this genetic screen<sup>26</sup> is based on a fusion between the yeast Ade2p  
104 reporter protein and a GAR domain of 43 amino acid (43GAR). Because GAR is able to self-inhibit  
105 the translation of its own mRNA in yeast, this leads to a reduction in Ade2p level. This can easily  
106 be monitored in yeast as cells which express Ade2p at a functional level form white colonies,  
107 whereas cells that do not express Ade2p readily form red colonies and any intermediate level of

4

108 Ade2p leads to the formation of pink colonies whose intensity of coloration is inversely  
109 proportional to the level of Ade2p expressed. Hence, a yeast strain expressing the *43GAR-ADE2*  
110 construct from the constitutive *ADH* promoter forms pink colonies, whereas a control strain  
111 expressing *ADE2* from the same promoter forms white colonies (**Fig. 1a**). We used the *43GAR-*  
112 *ADE2* strain to identify yeast genes whose overexpression leads to a redder phenotype meaning  
113 that they potentially exacerbate GAR-based inhibition of translation. For this purpose we  
114 transformed the *43GAR-ADE2* yeast strain by a yeast genomic DNA library consisting of small  
115 genomic fragments (~4kb) cloned into a yeast 2  $\mu$  multicopy plasmid which is present at ~50 to  
116 100 copies per yeast cell, hence potentially allowing to assess the effect of overexpressing every  
117 yeast gene on GAR-based inhibition of translation. This way, we isolated two independent clones  
118 bearing overlapping genomic fragments that, among a few other genes, contained the yeast  
119 *NSR1* gene. We then subcloned *NSR1* gene alone under the control of the strong constitutive  
120 *GPD* promoter in a low copy number vector (CEN) and confirmed its ability, when  
121 overexpressed, to both confer a redder phenotype and exacerbate the ability of GAR to decrease  
122 *43GAR-Ade2p* protein expression whereas having no significant effect on Ade2p protein in the  
123 control strain (**Fig. 1b** and **Supplementary Fig. 1a**) nor on *43GAR-ADE2* or *ADE2* mRNA levels  
124 (**Supplementary Fig. 1b**).

125 We then determined the effect of *NSR1* downregulation on GAR-based inhibition of protein  
126 expression. As *NSR1* is not essential in yeast, we deleted this gene in both *43GAR-ADE2* and *ADE2*  
127 strains and observed that its absence completely abolished the GAR inhibitory effect on *43GAR-*  
128 *Ade2p* expression (first two lanes of the western blot in **Fig. 1c**). This effect was GAR-dependent  
129 as *NSR1* deletion had no effect on Ade2p protein level in the control strain (last two lanes of the  
130 western blot in **Fig. 1c**). As controls, we checked that *NSR1* deletion had no significant effect on  
131 *43GAR-ADE2* and *ADE2* mRNA levels (**Supplementary Fig. 1c**).

132 Next, we assessed the potential ability of NCL, the gene encoding the human nucleolin, to  
133 functionally complement the deletion of *NSR1* in yeast and found that the expression of NCL led

134 to a decrease in 43GAR-Ade2p level whereas having no effect on Ade2p level (**Fig. 1d**). Of note,  
135 the human NCL was as efficient as the yeast Nsr1p (**Supplementary Fig. 1d**).

136 Taken together, these results show that Nsr1p, the yeast orthologue of nucleolin, is critically  
137 involved in the GAR-based inhibition of protein expression in yeast. As NCL, the human nucleolin,  
138 is able to fully complement the deletion of the yeast *NSR1* gene, this suggests that NCL  
139 represents a host cell factor important for the GAR-mediated self-limitation of EBNA1 expression  
140 in EBV-infected human cells.

141

#### 142 **NCL also controls GAR-dependent EBNA1 expression in EBV-infected cells**

143 We next assessed the role of human nucleolin (NCL) on GAR-based self-inhibition of translation  
144 in human cells. For this purpose, we first overexpressed HA-tagged NCL (HA-NCL) in three EBV-  
145 infected B cell lines: Mutu-1, B95.8 and Raji. As shown in **Figure 2a**, overexpression of HA-NCL  
146 led to a significant decrease in EBNA1 endogenous level in all these three cell lines, as compared  
147 to actin. Similar to yeast, this effect is GAR-dependent as overexpression of HA-NCL also  
148 decreased the level of transfected 235GAR-OVA (ovalbumin), the fusion protein that is used to  
149 assess the effect of GAR on MHC class I-restricted antigen presentation (see below), whereas  
150 having no significant effect on OVA alone, the control protein (**Supplementary Fig. 2a**).

151 We then determined the effect of downregulating endogenous NCL on GAR-dependent  
152 suppression of protein expression in H1299 cells using siRNA. As observed by others<sup>27</sup>, we were  
153 not able to knockdown more than ~40-50% the expression of NCL, probably because it is an  
154 essential gene in mammalian cells. However, this partial downregulation led to a significant  
155 increase in both EBNA1 (**Fig. 2b**) and 235GAR-OVA (**Fig. 2c**) protein levels, whereas having no  
156 effect on EBNA1ΔGAR or on OVA alone. Quantifications of three independent experiments are  
157 shown in **Supplementary Figure 2b** and **c**. Next we performed a metabolic <sup>35</sup>S methionine pulse-  
158 labeling experiment, and we observed an increase in newly synthesized 235GAR-OVA following  
159 NCL down-regulation (**Fig. 2d**, upper panel). In contrast, no increase in newly synthesized OVA

160 was observed (**Fig. 2d**, lower panel). We did not observe any significant effect on the levels of  
161 GAR-carrying mRNAs (**Supplementary Fig. 2d** and **2e** respectively). Altogether, these results  
162 demonstrate that NCL downregulation does interfere with GAR-based suppression of translation.  
163 These findings confirmed that, as in yeast, NCL represents a host cell factor critically involved in  
164 the GAR-dependent suppression of EBNA1 synthesis, a mechanism at the basis of EBV immune  
165 evasion in latently infected cells.

166

#### 167 **NCL downregulation overrides GAR-restricted antigen presentation**

168 We next tested if downregulating NCL has an effect on GAR-restricted MHC class I antigen  
169 presentation. Indeed, as NCL downregulation led to a GAR-dependent increase in protein  
170 expression, it was also expected to stimulate antigen presentation. For this purpose, we  
171 determined the effect of siRNA-mediated NCL knockdown on the GAR-restricted presentation of  
172 the ovalbumin-derived SIINFEKL antigenic peptide (OVA<sub>257-264</sub>) in complex with the murine k<sup>b</sup>  
173 MHC class I receptor using a specific monoclonal antibody. FACS analysis revealed that NCL  
174 knockdown significantly increased (+ 42.1% ± 1.37, p=0.0005) the formation of this complex in  
175 235GAR-OVA-expressing cells (**Fig. 3a**). In contrast, NCL knockdown only had a modest effect on  
176 OVA-expressing cells (+ 19.7% ± 6.11, p=0.043; **Fig. 3b**). In all cases, the efficiency of siRNA-  
177 mediated NCL downregulation and its effect on 235GAR-OVA or OVA expression were  
178 determined (**Supplementary Fig. 3a**).

179 We then tested if the observed increase in antigen presentation following NCL downregulation  
180 does have an effect on T cell activation. For this purpose, we determined the proliferation of  
181 naive CD8<sup>+</sup> T cells (OT1 cells) recognizing specifically the OVA<sub>257-264</sub> SIINFEKL epitope on the  
182 murine k<sup>b</sup> MHC class I molecule. The OT1 cells were isolated from peripheral and mesenteric  
183 lymph-nodes of 12-week-old mice and stained with the CellTrace™ Violet fluorescent dye. Then,  
184 OT1 cells were mixed with H1299 cells expressing 235GAR-OVA and the k<sup>b</sup> molecule. As a  
185 control, H1299 cells expressing OVA and the k<sup>b</sup> molecule were used. As expected and due to the

186 GAR inhibitory effect on both translation and antigen presentation, 235GAR-OVA-expressing  
187 H1299 cells (**Fig. 3c**, left panel) led to a much weaker activation of OT1 cells as compared to  
188 OVA-expressing H1299 cells (**Fig. 3d**, left panel), as determined by evaluating the number of  
189 dividing OT1 cells by FACS analysis. However, siRNA-mediated NCL knockdown in 235GAR-OVA-  
190 expressing H1299 cells significantly increased proliferation of OT1 cells (**Fig. 3c**, right panels)  
191 whereas it had no effect in OVA-expressing H1299 cells (**Fig. 3d**, right panels). The efficiency of  
192 siRNA-mediated NCL downregulation and its effect on 235GAR-OVA or OVA expression are  
193 shown in **Supplementary Fig. 3b**.

194

#### 195 **NCL directly interacts in the nucleus with G4 present in GAR-encoding mRNA sequence**

196 NCL has been reported to bind to some G-quadruplexes (G4) formed in both DNA<sup>24</sup> and RNA  
197 sequences<sup>28</sup>. G4 are composed and stabilized by the stacking of guanine tetrads which are  
198 assembled in a planar arrangement by Hoogsteen hydrogen bonding (**Fig. 4a**) and have been  
199 involved in the regulation of gene expression, DNA replication and telomere maintenance (for  
200 review<sup>20</sup>). The G-rich sequence of GAR-encoding mRNA contains a cluster of 13 predicted G4<sup>29</sup>.  
201 Hence, we determined the ability of NCL to bind to these structures. For this purpose, we  
202 adapted a pulldown assay recently developed to identify RNA G4 binding proteins<sup>28</sup> to an 18 nt-  
203 long oligonucleotide containing the most probable G4 that can form in the GAR-encoding mRNA  
204 sequence. Briefly, this oligonucleotide (GQ) was linked to biotin and pulldown experiments using  
205 streptavidin-conjugated sepharose beads were performed. As a negative control, we used an  
206 oligonucleotide (GM) with a similar sequence except that the four guanines forming the G4 were  
207 replaced by adenines or uridines in order to completely abolish the G4 structure, as predicted  
208 using the GQRS-H predictor software<sup>30</sup>. As a positive control, we used ARPC2 30 nt-long  
209 oligonucleotide which corresponds to a G4 found in the ARPC2 mRNA and that has been shown  
210 to bind NCL<sup>28</sup>. As shown in **Figure 4b**, NCL was precipitated from H1299 cell extracts when using

211 GQ or ARPC2 oligonucleotides, but not when using GM or empty beads showing that NCL binds  
212 to G4 formed in the GAR mRNA sequence.

213 Next we performed the same pulldown experiment using recombinant NCL instead of cell lysate.

214 Similar results were obtained (**Fig. 4c**) showing that NCL directly binds GAR most probable G4.

215 Finally, we wanted to check if, and where, the NCL-GAR G4 interaction occurs *in cellulo*. Hence,

216 we performed a proximity ligation assay (PLA) to assess if NCL associates with EBNA1 mRNA in

217 H1299 cells expressing EBNA1. We observed nuclear PLA dots in cells expressing the full-length

218 EBNA1 mRNA (**Fig. 4d**). In contrast, no dots were detected in cells expressing EBNA1ΔGAR as well

219 as in the various controls (**Fig. 4d**).

220 Taken together, these results indicate that NCL directly interacts with the G4 of the EBNA1

221 mRNA in the nuclear compartment.

222

#### 223 **PhenDC3 prevents NCL-EBNA1 mRNA interaction and GAR-based inhibition of protein** 224 **expression**

225 Next, we tested the effect of various G4 ligands on GAR-based inhibition of protein expression.

226 Among the reported G4 ligands, pyridostatin (PDS, molecular structure depicted in **Fig. 5a**) and

227 PhenDC3 (molecular structure depicted in **Fig. 5b**)<sup>31-33</sup> are the best benchmark probes

228 compatible with cellular assays. Indeed, PDS and PhenDC3 at micromolar concentrations have

229 been shown to efficiently target various G4 in cell-based experiments<sup>34</sup>. As, at the same range of

230 concentrations (1-5 μM), PDS has been shown to exacerbate GAR-based inhibition of protein

231 expression in an *in vitro* coupled transcription-translation system<sup>29</sup>, we first tested the effect of

232 this molecule on the level of 235GAR-OVA in H1299 cells. However, at the same concentration

233 (5 μM), no clear effect on 235GAR-OVA or OVA expression was observed (**Fig. 5a**) suggesting that

234 PDS may not be able to interfere with GAR-mediated inhibition of EBNA1 expression in a cellular

235 context. We then tested PhenDC3 at the same concentration (5 μM) and found that it led to a

236 significant increase in the steady-state level of 235GAR-OVA in H1299 cells (**Fig. 5b**, left panel).

237 This effect is GAR-dependent since PhenDC3 had no significant effect on OVA expression (**Fig. 5b**,  
238 right panel) and is not due to an effect on the level of the corresponding RNA (**Fig. 5c**). Hence,  
239 one possibility is that PhenDC3 prevents the binding of NCL on EBNA1 mRNA G4. To test this  
240 hypothesis, we performed the same G4 oligonucleotide pulldown assay as in **Figure 4b** in the  
241 presence or absence of 10  $\mu$ M PhenDC3 and we observed that PhenDC3 does prevent the  
242 binding of NCL on GAR G4 (**Fig. 5d**), readily explaining its effect on 235GAR-OVA expression.  
243 Next, we tested PhenDC3 effect on EBNA1 expression and found that it also increased the  
244 endogenous EBNA1 level in Mutu-1 (EBV-infected B-cells; **Fig. 5e** left panel) and NPC-6661 (EBV-  
245 infected cells derived from a nasopharyngeal carcinoma<sup>35</sup>; **Fig. 5e** right panel) cells. Importantly,  
246 PhenDC3 had no effect on EBNA1 mRNA level in Mutu-1 cells (**Supplementary Fig. 3c**).  
247 To conclude, the PhenDC3 G4 ligand prevents the binding of NCL on GAR's G4 and, at the same  
248 time, leads to an increase in EBNA1 and 235GAR-OVA expression, thereby supporting the crucial  
249 role of NCL in GAR-based self-inhibition of translation by binding to G4 formed in the EBNA1  
250 mRNA. Importantly, these results also indicate that NCL-EBNA1 mRNA interaction is druggable.

251

#### 252 **PhenDC3 activates GAR-limited antigen presentation**

253 Finally, to assess the ability of PhenDC3 to interfere with GAR-based immune evasion, we  
254 performed the same OT1 T cell proliferation assay as in **Figure 3c & d** in the presence or absence  
255 of 5  $\mu$ M PhenDC3. As shown in **Figure 6a**, PhenDC3 significantly increased (two-fold change) the  
256 proliferation of OT1 T cells added to 235GAR-OVA-expressing H1299 cells whereas it had no  
257 effect on OT1 cells added to OVA-expressing cells. A western blot analysis confirmed that  
258 PhenDC3 at 5  $\mu$ M increases the level of 235GAR-OVA whereas it has no effect on OVA (**Fig. 6b**).

259

#### 260 **DISCUSSION**

261 In this study we have identified nucleolin as the first host cell factor critically involved in GAR-  
262 based EBNA1 immune evasion *via* its ability to bind G-quadruplexes formed in the GAR-encoding

263 sequence of the EBNA1 mRNA. First, thanks to a genetic screen performed in the yeast model  
264 that recapitulates all the aspects of GAR-based self-inhibition of translation <sup>26</sup>, we isolated the  
265 yeast nucleolin Nsr1p as a critical host cell factor involved in GAR-based inhibition of protein  
266 expression in yeast. Indeed, the overexpression of Nsr1p exacerbates the GAR effect whereas  
267 the deletion of *NSR1* gene fully abrogates GAR ability to self-inhibit translation. Second, as the  
268 human NCL gene, which encodes human nucleolin, is able to fully complement the effect of  
269 *NSR1* deletion on GAR in yeast, we tested the role of NCL in GAR-based inhibition of protein  
270 expression in human cells and found that its overexpression and downregulation had the same  
271 effect on GAR than in yeast cells. Then we checked that, in line with its ability to interfere with  
272 GAR-mediated translation inhibition, NCL downregulation also increases antigen presentation  
273 and T cell proliferation. We also showed that NCL directly interacts with G4 present in GAR-  
274 encoding sequence of the EBNA1 mRNA *in vitro* and observed this interaction *in cellulo* in the  
275 nucleus. This suggests that the binding of NCL on EBNA1 mRNA G-quadruplexes is *per se*  
276 important for GAR-based translation inhibition and thereby for EBNA1 immune evasion. In line  
277 with this model, we showed that the G4 ligand PhenDC3 both prevents the binding of NCL on G4  
278 of the EBNA1 mRNA, and increases EBNA1 expression and GAR-dependent antigen presentation.  
279 This indicates that the interaction between NCL and G4 of the EBNA1 mRNA is a relevant and  
280 druggable therapeutic target to treat EBV-related cancers. Interestingly the G4 ligand  
281 pyridostatin (PDS) had no effect on EBNA1 expression *in cellulo* indicating that only some G4  
282 ligands are able to interfere with NCL-EBNA1 mRNA interaction, which can be attributed to off-  
283 target binding and/or differences in pharmacological properties (cell penetration, intracellular  
284 distribution etc.). This also points out two different possible mechanisms of action for G4 ligands  
285 whose binding on G4 may either stabilize them or prevent the binding of cellular partners, or  
286 both. In line with these hypotheses, we tested several PhenDC3 close chemical derivatives and  
287 observed that some, but not all, were active on EBNA1 expression (data not shown). Of note,  
288 PDS has been found to suppress EBNA1 expression in an *in vitro* coupled transcription-

289 translation assay<sup>29</sup> but it is not known, in this experiment, if this effect is related to a change in  
290 EBNA1 mRNA level or to G4 stabilization that may exacerbate the GAR-dependent translation  
291 inhibition.

292 What could be NCL mechanism of action in GAR-based self-inhibition of translation and antigen  
293 presentation? EBNA1 G4 may constitute a recognition signal for NCL that is, itself, directly or  
294 indirectly, responsible for translation inhibition by interfering with either translation initiation  
295 and/or elongation machinery. Alternatively, NCL could stabilize G4 that, themselves, may inhibit  
296 the ribosome progression. Of note, either of these two possible mechanisms would explain why  
297 EBNA1 mRNA is translated mainly in mitosis, at a time when the nuclear envelope has been  
298 disaggregated<sup>4,25</sup>. Finally, NCL could act by trapping EBNA1 mRNA in the nucleus given that NCL  
299 is a nuclear protein, thereby preventing its efficient translation by the ribosome. Importantly,  
300 these mechanisms are not mutually exclusive and a model in which NCL would inhibit  
301 translation by preventing ribosome progression during mitosis and by trapping EBNA1 mRNA in  
302 the nucleus during the other phases of the cell cycle can be envisioned. In either case, it is  
303 unlikely that the virus has developed a novel mechanism to exploit nucleolin for controlling gene  
304 expression. Rather, it is likely that this reflects a more general evolutionary conserved cellular  
305 pathway. The fact that NCL effect on GAR-based limitation of protein expression is also operant  
306 in yeast strengthens this hypothesis as yeast has no common evolutionary history with EBV.  
307 Hence, PhenDC3 and more generally G4 ligands represent chemical probes that will greatly help  
308 to characterize this pathway and to identify its physiological mRNA targets. To finish, as other  
309 oncogenic gammaherpesviruses like the Kaposi sarcoma-associated herpesvirus have evolved  
310 similar strategy of G4 clustering in the coding regions of their GMP to evade the immune  
311 system, the results presented here may have applications for other gammaherpesviruses-  
312 related diseases<sup>29,36</sup>.

313

314

315 **METHODS**316 **Yeast strains and culture media**

317 All the yeast strains used in this study are derived from the W303 *WT* strain<sup>37</sup>: *MATa, leu2-3,112*  
318 *trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*. The *ade2Δ* strain genotype is: *MATa, leu2-3,112 trp1-*  
319 *1 can1-100 ura3-1 ade2-1::his5 S. pombe*. Yeast cells were grown and used as previously  
320 described<sup>38</sup>. The media used for yeast growth were: YPD [1% (w/v) yeast extract, 2% (w/v)  
321 peptone, 2% (w/v) glucose], <sup>1/3</sup> YPD [0.33% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v)  
322 glucose]. Yeast minimal media w/o uracil [6.7% (w/v) yeast nitrogen base, 0.77% (w/v) amino  
323 acids without uracil, 2% (w/v) glucose]. Yeast minimal media w/o uracil and tryptophan [6.7%  
324 (w/v) yeast nitrogen base, 0.72% (w/v) amino acids without uracil, 2% (w/v) glucose]. Solid  
325 media contained 2% (w/v) agar.

326

327 **Yeast-based genetic screen for isolation of enhancers of GAR-based translation inhibition**

328 A yeast genomic DNA library (a kind gift by F. Lacroute) constructed by inserting ~4 kb genomic  
329 DNA fragments (obtained by *Sau3A* partial digestion) at the unique BamHI site in the replicative  
330 2 μ multicopy pFL44L vector containing *URA3*-marker, was transformed into 43GAR-Ade2p pink  
331 yeast strain using standard lithium acetate procedures<sup>39</sup>. This multicopy plasmid is present at ~  
332 50-100 copies per yeast cell. Transformants were selected on uracil-free minimal solid medium  
333 and a positive selection was carried out based on the redder color phenotype. Out of ~20,000  
334 transformants growing on uracil-free medium, 39 gave a redder phenotype. Plasmids originated  
335 from the pFL44-based library were extracted from these 39 redder transformants, purified and  
336 amplified in *E. coli* and then re-transformed into 43GAR-Ade2p yeast strain for confirmation of  
337 the redder phenotype. The extremities of the confirmed clones were sequenced using the  
338 following primers: F- 5' GTGCTGCAAGGCGATTAAGT 3' and R- 5'TGTGGAATTGTGAGCGGATA 3'.  
339 Two confirmed clones contained overlapping genomic fragments containing the yeast *NSR1*  
340 gene.

341

342 **Plasmid constructions**

343 All vectors were constructed using standard cloning procedures. T4 DNA ligase and restriction  
344 enzymes were purchased from New England Biolabs. Plasmid maintenance was carried out in  
345 TOP10 *E.coli* strain. The p416 (*GPD*) containing *NSR1* gene was constructed as follows: *NSR1*  
346 coding sequence was amplified from genomic DNA of the *S. cerevisiae* W303 WT strain using the  
347 following primers: NSR1-F 5' CGCGGATCCATGGCTAAGACTACTAAAGTAAAAGGTAAC 3' and NSR1-  
348 R 5' CCGCTCGAGCGGTTAATCAAATGTTTTCTTTGAACCAG 3'. The corresponding PCR fragment was  
349 cloned into *BamHI* and *XhoI* cloning sites of p416 (*GPD*) centromeric vector. In order to  
350 introduce a HA tag in frame with human *NCL*, its coding sequence was PCR-amplified from cDNA  
351 extracted from HEK293T cells using HA-NCL F- 5'  
352 CGCGGATCCATGTACCCATACGATGTCCAGATTACGCTGTGAAGCTCGCGAAGGCAG 3' and NCL R- 5'  
353 CCGCTCGAGCGGCTATTCAAACCTCGTCTTCTTTCC 3' primers and cloned into pCDNA3 vector  
354 (Invitrogen) using *BamHI* and *XhoI* cloning sites. HA-NCL was then sub-cloned into the  
355 *S.cerevisiae* vector p414 (*GPD*). All generated constructs were amplified in the TOP10 *E.coli*  
356 strain, and sequenced by the Sanger method.

357

358 **Generation of *nsr1Δ* yeast strains**

359 *NSR1* gene deletion was carried out by replacement with kanMX6 cassette amplified from  
360 PFA6a-kanMX6 vector<sup>40</sup>, using the following primers: F-5'  
361 ACCAATTTCCGGATCACTCAACCCAGGCAGGATAAAATAAGCGGATCCCCGGGTTAATTAA 3' and R-5'  
362 AAGAGAAAAAATTGAAATTGAAATTCATTTCTCAGAATCCGAGCTCGTTTAAAC 3'. Then the  
363 PCR fragment was transformed into W303 *ade2Δ*, *43GAR-ADE2* and W303 *ade2Δ*, *ADE2* yeast  
364 strains using standard lithium acetate procedures<sup>39</sup>. The transformed cells were spread on  
365 YPD + 100 µg/mL kanamycin plates which were then incubated 5 days at 29°C, after which the  
366 plates were replicated on fresh YPD + 100 µg/mL kanamycin plates, and the deletion of *NSR1*

14

367 gene in kanamycin-resistant colonies was checked by PCR, using the following primers: *nsr1Δ*  
368 Fbis-5' GTACTTAAGTGCTAGCTGTTGC 3' and *nsr1Δ* Rbis-5' TAGAGATGGTGAATGAAAGG 3'.

369

#### 370 **Yeast protein extracts**

371 5 mL of 0,8-1,0 OD<sub>600nm</sub> exponentially growing cells were harvested and cell pellets were  
372 resuspended into 300 μL of lysis buffer (25 mM Tris-HCl pH 6.8; 10% glycerol; 5% β-  
373 mercaptoethanol; 5% SDS; 8 M Urea; 0.02 % Bromophenol Blue).

374

#### 375 **Mammalian cells protein extracts**

376 Whole cells were harvested 48 hours post-transfection and lysed in 20 mM Tris-HCl, pH 7.5, 150  
377 mM NaCl, 1% Igepal containing protease inhibitors (Roche, Germany). Samples were centrifuged  
378 at 16,000 g during 20 min at 4°C and protein concentrations were measured using a Bradford  
379 assay.

380

#### 381 **Western blotting**

382 Equal protein quantities and volumes of all samples were loaded onto 10% NuPAGE® Bis-Tris  
383 gels (Invitrogen), and transferred onto 0.45 μm nitrocellulose membranes (GE Healthcare).  
384 Membranes were blocked during 1 hour at room temperature in PBS 1X containing 0.1% Igepal  
385 and 3% BSA.

386 Membranes were analyzed using the following antibodies: anti-HA serum (1:2500); anti-Nsr1p  
387 mouse monoclonal antibody (Abcam, 1:5000), anti-NCL rabbit polyclonal antibody (Abcam,  
388 1:5000), anti-GAPDH (Sigma, 1:5000), anti-EBNA1 mouse monoclonal antibody (OT1X, 1:2000),  
389 anti-OVA rabbit polyclonal antibody (Sigma, 1:2500), anti-actin (Sigma, 1/5000). The membranes  
390 were then washed with fresh PBS 1X + 0.1% Igepal and incubated for 45 min with swine anti-  
391 rabbit or goat anti-mouse secondary antibodies (Dako) conjugated to horseradish peroxidase at  
392 a 1:3000 dilution, and analyzed by enhanced chemiluminescence (ECL, GE Healthcare) using a

393 Vilbert-Lourmat Photodocumentation Chemstart 5000 imager. All the experiments were  
394 repeated at least three times. Relative protein levels for each sample were normalized to  
395 GAPDH or Actin protein levels as indicated, using Fusion-Capt Advance software.

396

#### 397 **Cell culture, Transfection.**

398 H1299 cells are derived from metastatic lymph node from lung carcinoma. Raji cells are type III  
399 latency Burkitt's lymphoma. HCT116 cells are derived from colorectal carcinoma. B95.8 cells are  
400 derived from cotton-top Tamarin Monkey peripheral blood lymphocyte. Mutu-1 cells are  
401 derived from an EBV-positive Burkitt's lymphoma biopsy specimen from a Kenyan patient. NPC-  
402 6661 cell line was established from a xenografted NPC in the early 90's<sup>35</sup> and was kindly  
403 provided by Prof. Kwok-Wai Lo from the Chinese University of Hong Kong. H1299, Raji, B95.8  
404 and Mutu-1 cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum  
405 (FBS) and 2 mM L-glutamine. HCT116 cells were cultured in McCoy's 5A Glutamax media  
406 supplemented with 10% FBS, and NPC-6661 cells in RPMI-1640 media supplemented with 25  
407 mM HEPES (Gibco) and 2 mM L-glutamine and 10% FBS. All cells were cultured at 37°C with 5%  
408 CO<sub>2</sub>. Transient transfections were performed using Genejuice reagent (Merck Bioscience)  
409 according to the manufacturer's protocol or electroporation using Gene PulserXL system  
410 (Biorad).

411

#### 412 **RNA extraction and quantitative real-time PCR**

413 Total yeast, H1299 and Mutu-1 cellular RNA was extracted using RNAeasy and RNAase-free,  
414 DNase kits (Qiagen). cDNA synthesis was carried out using 1 µg of DNA-free RNA using M-MLV  
415 reverse transcriptase (Invitrogen) and Oligo-dT primer. Triplicated cDNA samples were  
416 analyzed by quantitative PCR using PERFECTA SYBR fastmix (Quanta Bioscience). The relative  
417 abundance of target mRNA was normalized using Actin as an endogenous control.  
418 Quantification of gene expression was determined using the  $-2^{\Delta\Delta Ct}$  method. The primers used for

419 PCR were *ADE2*-forward: 5'-ATTGTGCAAATGCCTAGAGGTG-3', *ADE2*-reverse: 5'-AATCATAA -  
420 GCGCCAAGCAGTC-3'; Actin-forward: 5'-ATGGTNGGNATGGGNCARAAR-3', Actin-reverse: 5'-  
421 CTCCATRTCRTCCAGTTGGT-3'; *EBNA1*-forward: 5'-GGCAGTGGACCTCAAAGAAGAG-3'; *EBNA1*-  
422 reverse: 5'-CAATGCAACTTGGACGTTTTT-3'; OVA-forward: 5'-GAGGAGGCTTGGAACTAT-3'; OVA-  
423 reverse: 5'-CAGTTTGAGAATCCACGGAG-3'. All the experiments were performed in triplicates and  
424 were repeated at least three times.

425

#### 426 **NCL siRNA downregulation**

427 1, 75 x 10<sup>5</sup> H1299 cells were transfected with 0.75 µg of EBNA1, EBNA1ΔGAr, 235GAr-OVA or  
428 OVA vectors using standard procedures and incubated at 37°C for 8 hours. Cells were then  
429 transfected either with 40 nM of control siRNA or FlexiTube GeneSolution for NCL (Qiagen).  
430 siRNA transfection were performed using HiPerFect transfection reagent (Qiagen) following the  
431 manufacturer's protocol. 40 hours after siRNA transfection, cells were collected for western blot  
432 or flow cytometry analyses.

433

#### 434 **Flow cytometry analysis**

435 48 hours after the transfection, cells were collected using trypsin and washed twice with 1X PBS.  
436 Cells were suspended in 50 µL of 1X PBS and incubated with 0.4 µg of anti-Mouse OVA257-264  
437 (SIINFEKL) peptide antibody bound to H-2Kb PE or Anti-Mouse MHC Class I (H-2Kb) antibody  
438 bound to PE (Ebioscience) for 30 min at room temperature. Cells were then washed with 1X PBS  
439 and analyzed by FACS on a CANTO II flow cytometer (BD Biosciences, USA).

440

#### 441 **RNA pull-down experiments**

442 For the preparation of whole cell extracts, confluent H1299 cells were harvested after trypsin  
443 treatment and washed twice with 1X PBS (Gibco). Cells were suspended in 500 µL of lysis buffer  
444 (20 mM Tris-HCl pH 7.5; 200 mM NaCl and 0.1 % Igepal) containing 1X protease inhibitor cocktail

445 (Roche). Cell lysis was performed by 5 series of vortex followed by 10 min incubation on ice, and  
446 3 series of 3 sec sonication at 20% amplitude. After lysis cells were centrifuged at 4°C for 15 min  
447 at 16,000g, and the supernatant was quantified by Bradford. The whole cell extracts or  
448 recombinant GST-NCL (Abnova) were used for pull-down assays with the following G-quadruplex  
449 forming oligonucleotides: GQ- 5'-GGGGCAGGAGCAGGAGGA-3'Biotin TEG, ARPC2- 5'  
450 AGCCGGGGCUGGGCGGGGACCGGGCUUGU-3'Biotin TEG. The negative control for EBNA1 G-  
451 quadruplex was the GM- 5' GAGGCAGUAGCAGUAGAA-3'Biotin TEG oligonucleotide which,  
452 according to the GQRS-H predictor software <sup>30</sup>, is unable to form G4 structures. To avoid  
453 unspecific binding, high-affinity streptavidin sepharose beads (GE Healthcare) were incubated in  
454 1 mL blocking buffer containing 10 mM Tris-HCl pH 7.5; 100 mM KCl; 0.1 mM EDTA; 1 mM DTT;  
455 0.01% Triton X-100; 0.1% BSA; 0.02% *S. cerevisiae* tRNAs (Sigma), for 1 hour at 4°C on a rotating  
456 wheel. 10 pg of each folded biotinylated RNA oligos were incubated with 50 µL of solution  
457 containing the streptavidin sepharose beads for 90 min at 4°C on a rotating wheel. 500 µg of  
458 cell extract or 200 ng of recombinant GST-NCL were incubated with the RNA oligonucleotides  
459 bound to the streptavidin beads during 90 min at room temperature. Beads were washed with  
460 increasing KCl concentration (200-800 mM). Protein still bound to beads after the washes were  
461 eluted using 2X SDS loading buffer and analyzed by western blotting against NCL, as previously  
462 described.

463

#### 464 **Proximity Ligation Assay (PLA)**

465 Cells were fixed with 4% paraformaldehyde in PBS 1X for 20 min and permeabilized with 0.4%  
466 Triton X-100, 0.05% CHAPS for 5 minutes at room temperature. 50 ng of EBNA1-digoxigenin DNA  
467 probe (5' CTTTCAAACCACCTCCTTTTTGCGCCTGCCTCCATCAAAAA 3') were denatured 5  
468 minutes at 80 °C and the hybridization reaction was carried out overnight at 37 °C. A blocking  
469 solution of 3% BSA 0.1% saponine in 1X PBS was added for 30 min followed by 2 hours  
470 incubation with the primary antibodies (anti-digoxigenin 1/200 -Sigma- and anti-NCL 1/1000 -

471 Abcam-) diluted in 0,3% BSA, 0.1% saponine. The proximity ligation assay (PLA) was carried out  
472 using the Duolink PLA in situ kit (Sigma) following the manufacturer's protocol.

473

#### 474 **<sup>35</sup>S methionine pulse-labeling**

475 8 X 10<sup>5</sup> cells were transiently transfected with 4 µg of 235GAR-OVA or OVA vectors and, 8 hours  
476 later, NCL silencing was performed using 40 nM of NCL siRNA or control siRNA (as previously  
477 described). 40 hours after the transfection cells were incubated 30 min in a methionine-free  
478 medium. After incubation, 25 µM of MG132 proteasome inhibitor was added to the medium  
479 and cells were incubated for 45 min. Cells were then cultured in a medium containing 0.15  
480 mCi/mL <sup>35</sup>S-methionine (Perkin Elmer, Boston, USA) for 1 hour and harvested. Cell pellets were  
481 suspended in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal and treated as described above.  
482 Lysates were pre-cleared with 1 µg normal rabbit serum (Dako) bound to protein G-Sepharose  
483 magnetic beads (GE Healthcare) for 30 min at 4°C and further immunoprecipitated with 1 µg of  
484 anti-OVA polyclonal antibody (Sigma) or IgG-rabbit (Dako), pre-bound to protein G-Sepharose  
485 magnetic beads overnight at 4°C. Beads were then washed and proteins eluted using 2X SDS  
486 loading buffer. Immunoprecipitates were analyzed by SDS-PAGE using 10% precast NUPAGE gels  
487 (Invitrogen). The amount of radiolabeled proteins was visualized using a Storm Phosphorimager  
488 (GE Healthcare).

489

#### 490 **T cell proliferation assay**

491 Naive OVA<sub>257-264</sub> specific CD8<sup>+</sup> T cells were isolated by negative selection from peripheral and  
492 mesenteric lymph-nodes of 12-week-old female OT1 mice using the CD8<sup>+</sup> T cell isolation kit  
493 (Miltenyi Biotec, Germany). Afterwards, CD8<sup>+</sup> T cells were stained with CellTrace™ Violet  
494 (Thermo Fisher Scientific, USA) according to the manufacturer's protocol and mixed with H1299  
495 cells cotransfected with mouse k<sup>b</sup> expression vector and OVA or GAR-OVA constructs. For all the  
496 assays, 10<sup>5</sup> H1299 cells were harvested 48h after transfection and co-incubated with 4 x 10<sup>5</sup>

497 stained T cells at 37°C in humidified air/CO<sub>2</sub> atmosphere in 1 ml of RPMI medium containing 10%  
 498 FBS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 mM HEPES and 0.05 mM  
 499 2-mercaptoethanol (Sigma-Aldrich). After 3 days, cells were harvested, stained with hamster  
 500 anti-mouse CD3-APC (Miltenyi Biotec) and fixable viability dye eFluor® 780 (eBioscience, USA)  
 501 and analyzed by FACS on a CANTO II flow cytometer (BD Biosciences, USA). Cells were gated for  
 502 live CD3<sup>+</sup> cells (10.000 events collected) and data were analyzed using BD FACSDiva software  
 503 version 8.0.1. The percentage of proliferating T cells was considered for statistical analysis.

504

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614

#### 615 AUTHOR CONTRIBUTIONS

616 M.J.L., C.V., and M.B. conceived and designed the experiments. M.J.L., R.P.M., M.-A.C., S.F., P.P.,  
617 and C.D. performed the experiments. M.J.L., R.P.-M., M.-A.C., S.F., P.P., C.D., C.J., M.-P.T.-F., R.F.,  
618 C.V. and M.B. analyzed the data. M.P.T.-F., R.P.-M. and R.F. contributed reagents. M.J.L., R.F. and  
619 M.B. wrote the first draft of the paper. M.J.L., R.P.M., C.D., M.-P.T.-F., C.V., R.F. and M.B.  
620 reviewed and edited the manuscript. R.F., C.V. and M.B. supervised the work.

621

622 **COMPETING FINANCIAL INTERESTS**

623 The authors declare no competing financial interests.

624

625 **ADDITIONAL INFORMATION**

626 Supplementary information is available in the online version of the paper. Reprints and  
627 permissions information is available online. Correspondence and requests for materials should  
628 be addressed to M.B.

629

630 **ONLINE METHODS**

631

632

633 **FIGURE LEGENDS**

634 **Figure 1: Identification and confirmation of the critical role of nucleolin in GAR-based**  
635 **translation inhibition in yeast.**

636 **(a)** Rationale of the yeast-based genetic screen. Contrary to yeast cells expressing *ADE2* gene  
637 (left panel) that forms white colonies on rich medium (YPD), those expressing the *43GAR-*  
638 *ADE2* fusion (right panel) form pink colonies due to the ability of GAR to self-limit the  
639 translation of its own mRNA in yeast as in human cells. Based on this model, we looked for  
640 yeast genes whose overexpression from high copy number plasmids leads to a redder  
641 phenotype, suggesting an exacerbation of the GAR-based translation inhibition.

642 **(b)** Effect of *NSR1* overexpression on 43GAR-Ade2p protein level. The overexpression of *NSR1*  
643 gene, which encodes the yeast nucleolin, led to a redder phenotype associated to a  
644 decrease in 43GAR-Ade2p level as evidenced by SDS-PAGE and western blot analysis (left  
645 panel). GAPDH was used as a loading control. The mean 43GAR-Ade2p/GAPDH ratio from 3  
646 independent experiments is shown in the right panel.

647 **(c)** Effect of *NSR1* gene deletion on 43GAR-Ade2p and Ade2p protein level. SDS-PAGE and  
648 western blot analysis showing that deletion of *NSR1* (*nsr1Δ*) had no effect on Ade2p level,  
649 whereas it strongly increased the level of 43GAR-Ade2p. GAPDH was used as a loading  
650 control. The mean 43GAR-Ade2p/GAPDH or Ade2p/GAPDH ratios from 3 independent  
651 experiments are shown in the right panel.

652 **(d)** Human nucleolin (NCL) is able to complement *NSR1* deletion. SDS-PAGE and western blot  
653 analysis of the yeast *nsr1Δ* strain expressing 43GAR-Ade2p (left) or Ade2p (right) and  
654 overexpressing (right lanes), or not (left lanes), HA-tagged human nucleolin (HA-NCL).  
655 GAPDH was used as a loading control. The 43GAR-Ade2p/GAPDH or Ade2p/GAPDH ratio are  
656 indicated below the gels.

657

658 **Figure 2: Overexpression of NCL exacerbates GAR effect on protein expression whereas its**  
659 **downregulation reduces its inhibitory effect on translation.**

660 **(a)** SDS-PAGE and western blot analysis of the level of endogenous EBNA1 in three EBV-infected  
661 B cell lines overexpressing (right lanes), or not (left lanes), HA-tagged nucleolin (HA-NCL).

662 Actin was used as a loading control. EBNA1/actin ratios are indicated below the gels.

663 **(b)** SDS-PAGE and western blot analysis of the level of EBNA1 or EBNA1ΔGAR in response to NCL  
664 knockdown. H1299 cells were transfected with EBNA1 or EBNA1ΔGAR expressing vectors  
665 and with control siRNA or siRNA targeting NCL, as indicated. GAPDH was used as a loading  
666 control. EBNA1/GAPDH or EBNA1ΔGAR/GAPDH ratios are indicated below the gels. The  
667 efficiency of NCL downregulation was estimated by determining the level of remaining NCL  
668 in cells treated by siRNA targeting NCL versus cells treated by control siRNA.

669 **(c)** Same experiment as in **(b)** except that H1299 cells were transfected with chicken ovalbumin  
670 (OVA) or 235-GAR-OVA.

671 **(d)** Autoradiographs showing relative mRNA translation efficiencies of 235GAR-OVA versus OVA  
672 in response to NCL knockdown. H1299 cells transfected with 235GAR-OVA (upper panel) or  
673 OVA (lower panel) constructs and with control siRNA or siRNA targeting NCL, as indicated,  
674 were pulse-labelled with [<sup>35</sup>S] methionine, and lysates were subjected to  
675 immunoprecipitation with antibodies raised against OVA (left lanes) or IgG as a control (right  
676 lanes), and subjected to SDS-PAGE and autoradiography. Quantification of 235GAR-OVA or  
677 OVA signals are indicated.

678

679 **Figure 3: NCL downregulation activates GAR-limited antigen presentation and recognition by**  
680 **specific T lymphocytes.**

681 **(a)** Effect of NCL knockdown on antigen presentation. H1299 were transfected with 235GAR-OVA  
682 and murine MHC class I K<sup>b</sup> plasmids and with control siRNA or siRNA targeting NCL, as  
683 indicated, and the levels of Kb/OVA-derived antigenic peptide complexes determined using

684 FACS analysis. The quantification of the complex level obtained in cells treated by si-NCL as  
685 compared to cells treated by si-control is shown.

686 **(b)** Same experiment as in **(a)** except that H1299 cells were transfected with OVA plasmid.

687 **(c)** Effect of NCL knockdown on T cell proliferation. H1299 cells were transfected with mouse K<sup>b</sup>  
688 and 235GAR-OVA plasmids and control siRNA (left) or siRNA targeting NCL (right) and then  
689 mixed with naive OVA<sub>257-264</sub> (SIINFEKL) specific CD8<sup>+</sup> T cells isolated from peripheral and  
690 mesenteric lymph-nodes of mice and stained with CellTrace™ Violet. The proliferation of  
691 these SIINFEKL-specific T cells was determined by FACS analysis. Quantification of  
692 proliferating T lymphocytes when incubated in presence of cells treated by siRNA targeting  
693 NCL (si-NCL) as compared to T lymphocytes incubated with control siRNA-treated cells (si-  
694 control) is shown on the right.

695 **(d)** Same experiment as in (c) except that H1299 were transfected with OVA plasmid.

696

697 **Figure 4: NCL directly interacts with G4 formed in the GAR-encoding mRNA and NCL-**  
698 **EBNA1 mRNA interaction takes place in the nucleus.**

699 **(a)** Schematic representation of G4 structure. A guanine tetrad (G-tetrad) formed by the planar  
700 arrangement of four guanines held together by Hoogsteen hydrogen bonds (dashed lines)  
701 and stabilized by a cation (usually potassium) stacks with other G-tetrads to form G4.

702 **(b)** RNA pull-down using G4 forming RNA oligonucleotides covalently linked to biotin and  
703 streptavidin-coupled sepharose beads. Lysate from H1299 cells was applied to the following  
704 matrices: streptavidin-coupled beads either alone (Empty), or together with GQ (containing  
705 the most probable G4 of GAR mRNA), GM (same sequence except that G critical for G4  
706 formation were replaced by adenines or uridines) or ARPC2 (containing a G4 present in  
707 ARPC2 mRNA and that has been shown to bind NCL) RNA oligonucleotides. The sequence of  
708 these oligonucleotides is given in the **Methods** section. The proteins still bound to the beads  
709 after an 800 mM KCl wash were eluted and analyzed by SDS-PAGE and western blot.

710 (c) Same experiment as in (b) except that recombinant NCL was used instead of H1299 cell  
711 lysate.

712 (d) NCL interacts *in cellulo* with EBNA1 mRNA in a GAR-dependent manner and in the nucleus.  
713 Proximity ligation assay (PLA) experiment to determine if NCL interacts with EBNA1 mRNA *in*  
714 *cellulo*. The results for H1299 cells transfected with EBNA1 or EBNA1ΔGAR plasmids are  
715 shown. The red dots indicate an interaction. Mock-transfected cells are shown as a control,  
716 together with control cells without EBNA1-specific DNA probe or without NCL-specific  
717 antibodies.

718

719 **Figure 5: PhenDC3 prevents GAR inhibition of protein expression and NCL binding to GAR's G-**  
720 **quadruplexes.**

721 (a) SDS-PAGE and western blot analysis of the level of 235GAR-OVA or OVA in response to  
722 pyridostatin (PDS) treatment. H1299 cells transfected with 235GAR-OVA (left) or OVA  
723 (right) plasmids were treated with 5 μM PDS (right lanes) or, as a control, with DMSO  
724 (left lanes). GAPDH was used as a loading control and 235GAR-OVA/GAPDH and  
725 OVA/GAPDH ratios are indicated below the gels. The chemical structure of PDS is  
726 depicted on the right

727 (b) SDS-PAGE and western blot analysis of the level of 235GAR-OVA or OVA in response to  
728 PhenDC3 treatment. Same experiments as in (a) except that cells were treated with 5 μM  
729 PhenDC3 which chemical structure is depicted on the right.

730 (c) The effect of 5 μM PhenDC3 treatment on 235GAR-OVA or OVA mRNA level was  
731 determined by qRT-PCR.

732 (d) PhenDC3 competes for the binding of NCL on GAR G-quadruplexes. Same experiment  
733 than in **Figure 4 (c)** in the presence of 5 μM PhenDC3 (second and fourth lanes) or DMSO  
734 as a control (first and third lanes).

735           **(e)** PhenDC3 increases endogenous EBNA1 expression in EBV-infected cells. The level of  
736           endogenous EBNA1 in Mutu-1 (EBV-infected B cells, left panel) and NPC-6661 (EBV-  
737           infected cells from nasopharyngeal carcinoma, right panel) cells in response to 1  $\mu$ M  
738           PhenDC3 was determined by SDS-PAGE followed by western blot. Actin was used as a  
739           loading control and EBNA1/Actin ratio are indicated below the gels.

740

741           **Figure 6: PhenDC3 activates GAR-limited antigen presentation.**

742           **(a)** PhenDC3 increases T cell proliferation. Same experiment as in **Figure 3 (c) & (d)** except that  
743           235GAR-OVA (upper panels) and OVA (lower panels) expressing H1299 cells were treated  
744           with 5  $\mu$ M PhenDC3 or, as control, with DMSO as indicated. Quantification of proliferating T  
745           lymphocytes following PhenDC3 treatment as compared to DMSO-treated cells is shown in  
746           the graph on the right.

747           **(b)** SDS-PAGE and western blot analysis of cells used in **(a)**.

748

749 **SUPPLEMENTARY FIGURE LEGENDS**750 **Supplementary Figure 1: Identification and confirmation of the critical role of nucleolin in GAR-**  
751 **based translation inhibition in yeast.**

752 **(a)** Effect of *NSR1* overexpression on Ade2p level. The overexpression of *NSR1* gene, which  
753 encodes the yeast nucleolin, has no effect on the white color of *ADE2* expressing yeast cells  
754 and on the Ade2p protein level as evidenced by SDS-PAGE and western blot analysis (left  
755 panel). GAPDH was used as a loading control. The mean Ade2p/GAPDH ratio from 3  
756 independent experiments is shown in the right panel.

757 **(b)** Overexpression of *NSR1* has no effect on *43GAR-ADE2* and *ADE2* mRNA level in yeast. Relative  
758 levels of *43GAR-ADE2* or *ADE2* mRNA as compared to actin mRNA in *NSR1*-overexpressing  
759 cells were determined by quantitative RT-PCR.

760 **(c)** Deletion of *NSR1* has no effect on *43GAR-ADE2* and *ADE2* mRNA level in yeast. Relative levels  
761 of *43GAR-ADE2* or *ADE2* mRNA as compared to actin mRNA in *WT* or *nsr1Δ* cells were  
762 determined by quantitative RT-PCR.

763 **(d)** Complementation of *NSR1* deletion by the *NSR1* gene expressed from a plasmid. SDS-PAGE  
764 and western blot analysis of the yeast *nsr1Δ* strain expressing 43GAR-Ade2p (left) or Ade2p  
765 (right) and expressing (right lanes), or not (left lanes), yeast *NSR1*. GAPDH was used as a  
766 loading control. The 43GAR-Ade2p/GAPDH or Ade2p/GAPDH ratios are indicated below the  
767 gels.

768

769 **Supplementary Figure 2: Overexpression of NCL exacerbates GAR effect on protein expression**  
770 **whereas its downregulation suppresses its inhibitory effect on translation.**

771 **(a)** SDS-PAGE and western blot analysis of the level of 235GAR-OVA or OVA in response to the  
772 overexpression of NCL. HCT116 cells were transfected with 235GAR-OVA or OVA and with  
773 NCL plasmids, as indicated. GAPDH was used as a loading control. The OVA/GAPDH or  
774 235GAR-OVA/GAPDH protein level ratios are indicated below the gels.

775 **(b)**Quantification of the experiments shown in **Figure 2b**. The mean EBNA1/GAPDH and  
776 EBNA1ΔGAR/GAPDH protein level ratios from 3 independent experiments are shown.

777 **(c)**Quantification of the experiments shown in **Figure 2c**. The mean 235GAR-OVA /GAPDH and  
778 OVA/GAPDH protein level ratios from 3 independent experiments are shown.

779 **(d)**siRNA-mediated NCL knockdown has no effect on EBNA1 and EBNA1ΔGAR mRNA level in  
780 H1299 cells. H1299 cells were transfected with EBNA1 or EBNA1ΔGAR and with control  
781 siRNA or siRNA against NCL, as indicated. Relative levels of EBNA1 or EBNA1ΔGAR mRNA as  
782 compared to actin mRNA in cells treated with siRNA targeting NCL or control siRNA as  
783 indicated were determined by quantitative RT-PCR.

784 **(e)**Same experiment as in **(d)** except that H1299 cells were transfected with chicken ovalbumin  
785 (OVA) or 235GAR-OVA whose relative mRNA levels compared to actin mRNA were assessed  
786 by quantitative RT-PCR.

787

788 **Supplementary Figure 3: NCL downregulation activates antigen presentation and recognition**  
789 **by T lymphocytes.**

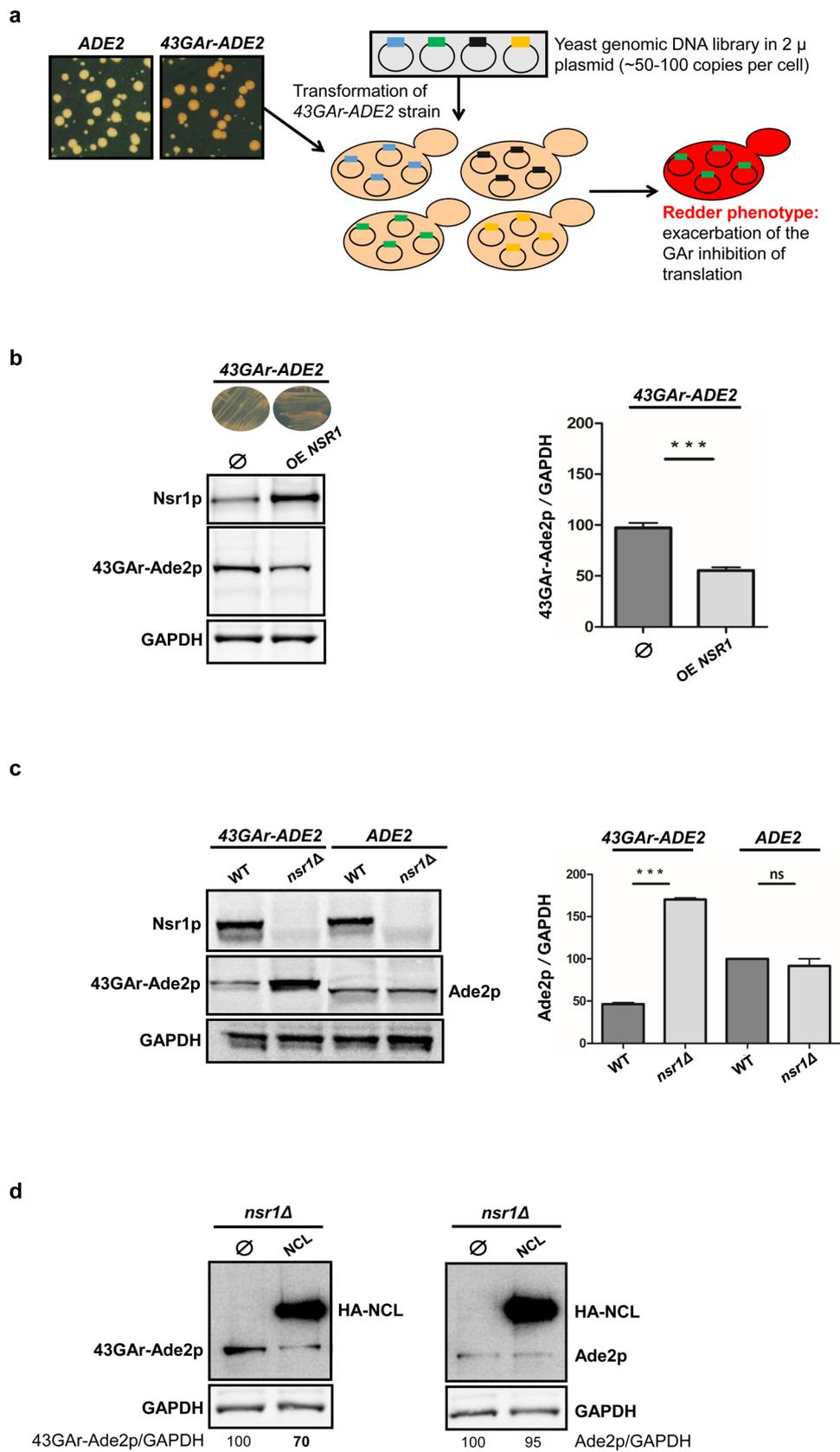
790 **(a)** SDS-PAGE and western blot analysis of the H1299 cells used in **Figure 3a & b**.

791 **(b)** SDS-PAGE and western blot analysis of the H1299 cells used in **Figure 3c & d**.

792 **(c)** Treatment with 5 μM PhenDC3 has no effect on EBNA1 mRNA level in Mutu-1 cells. Relative  
793 levels of EBNA1 mRNA as compared to actin mRNA in PhenDC3- or DMSO-treated cells were  
794 determined by quantitative RT-PCR.

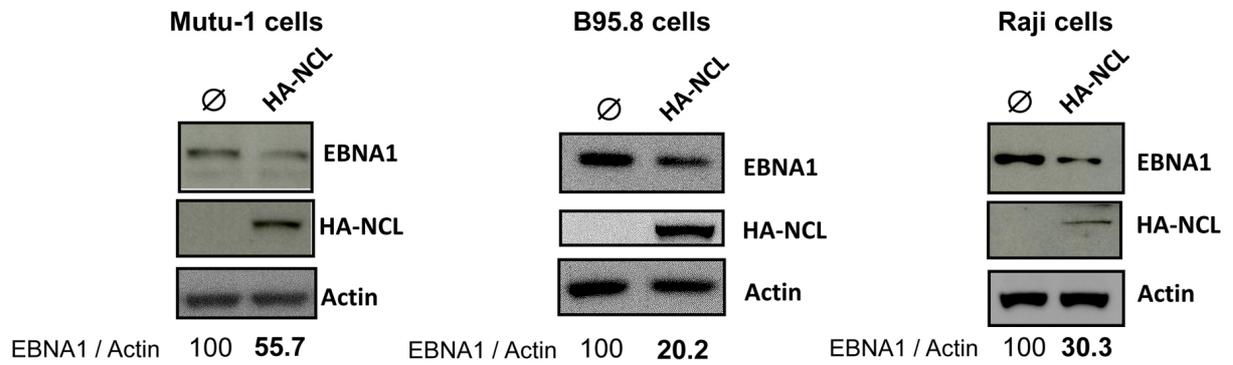
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Figure 1

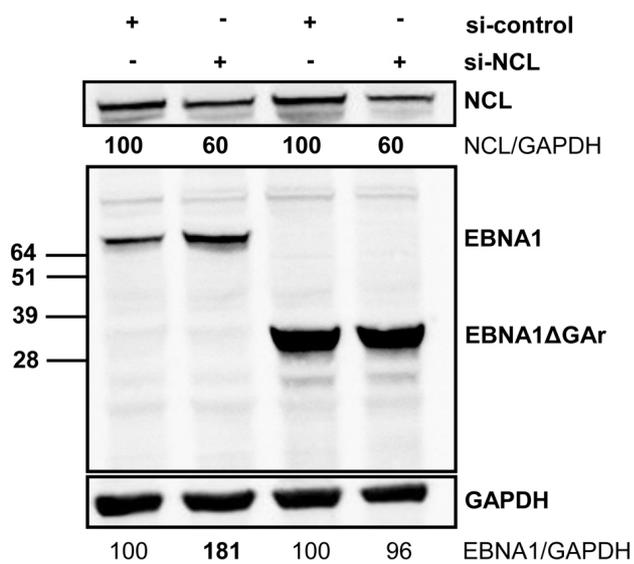


**Figure 2**

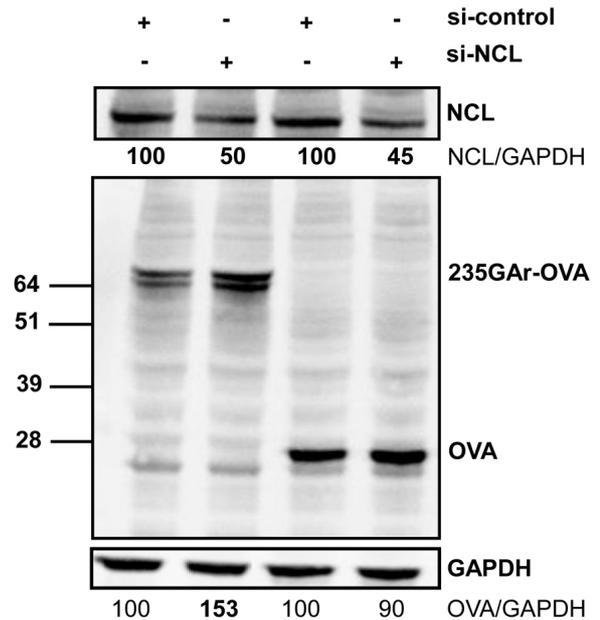
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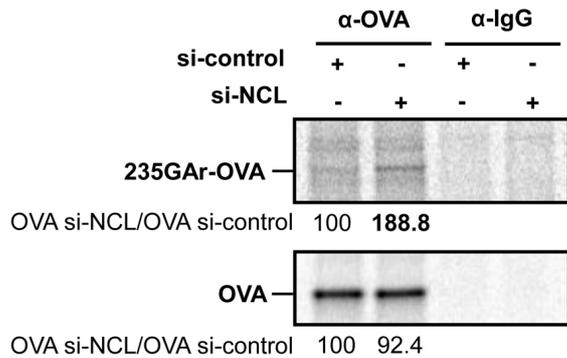
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**c**

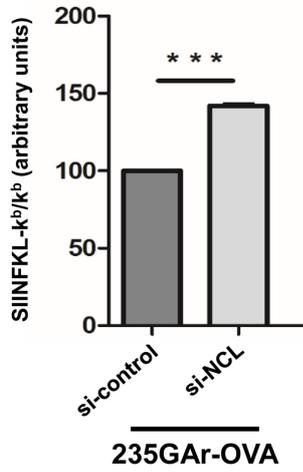


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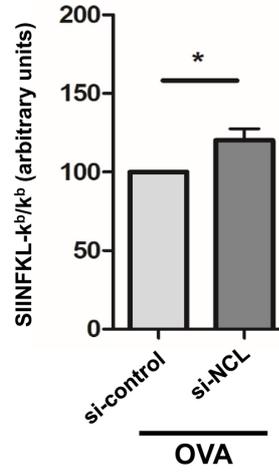


**Figure 3**

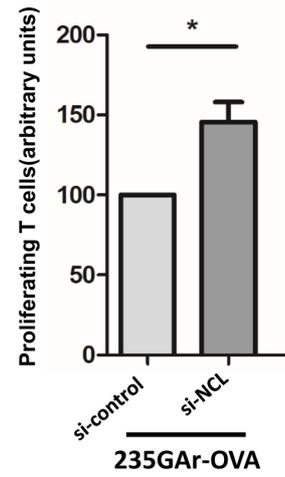
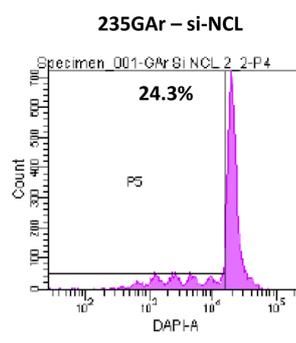
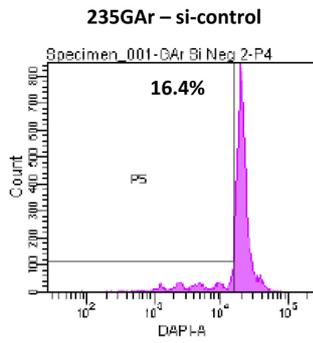
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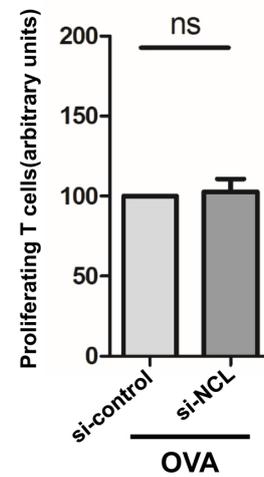
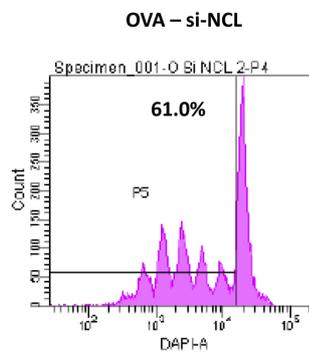
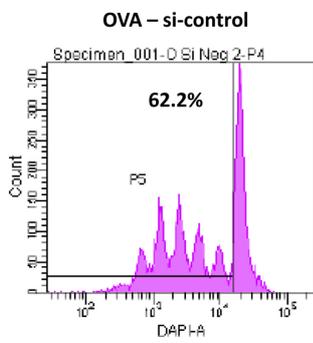
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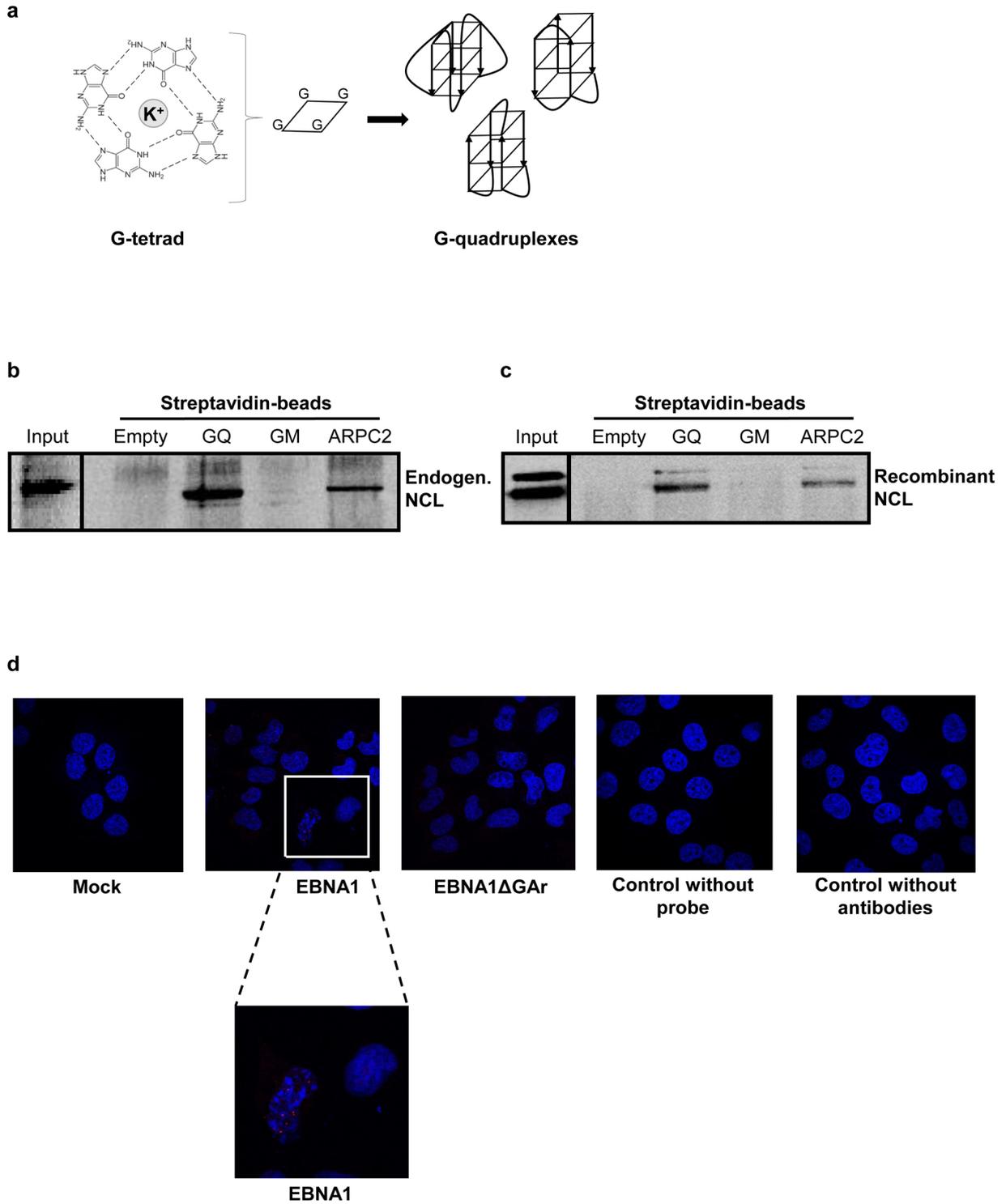
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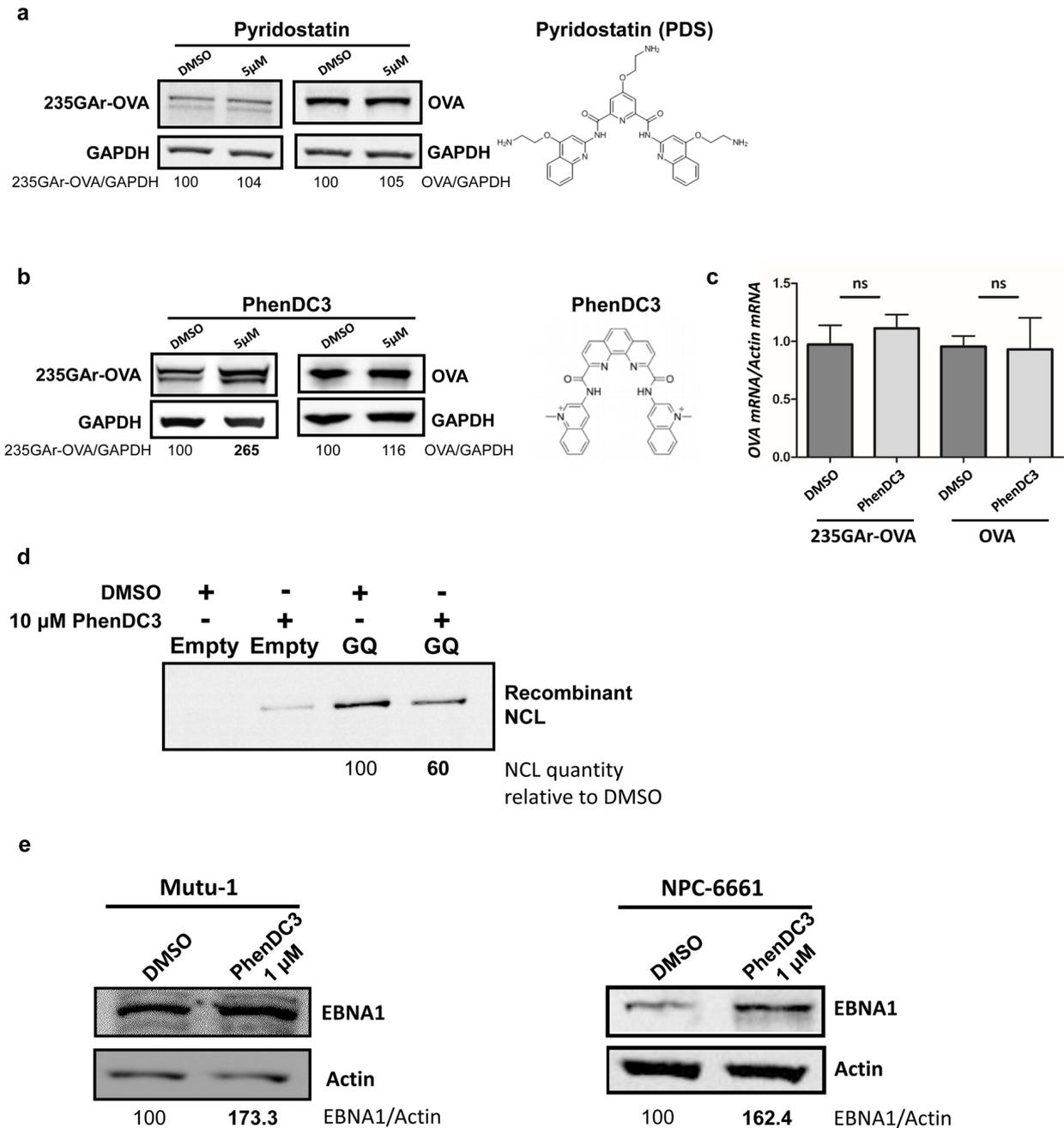
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**Figure 4**

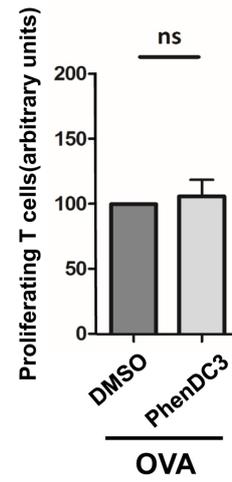
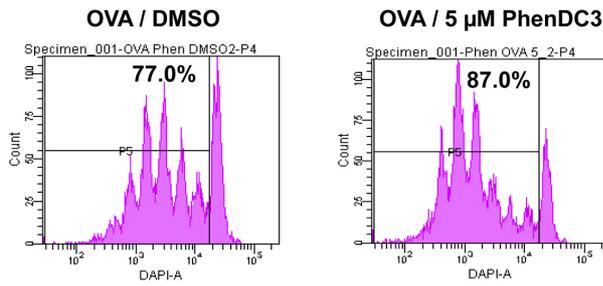
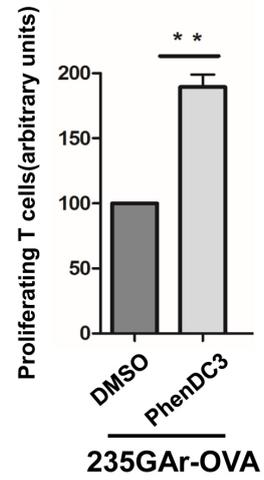
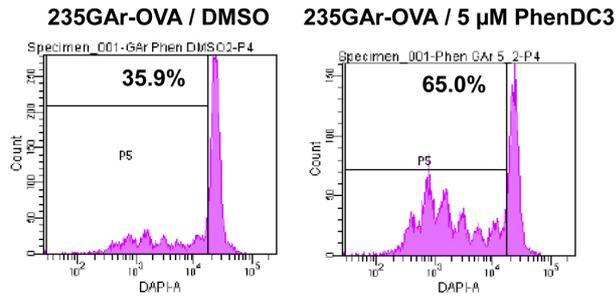


**Figure 5**

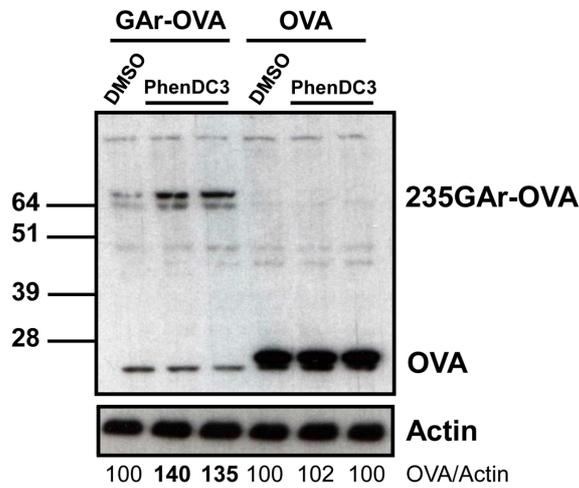


**Figure 6**

**a**

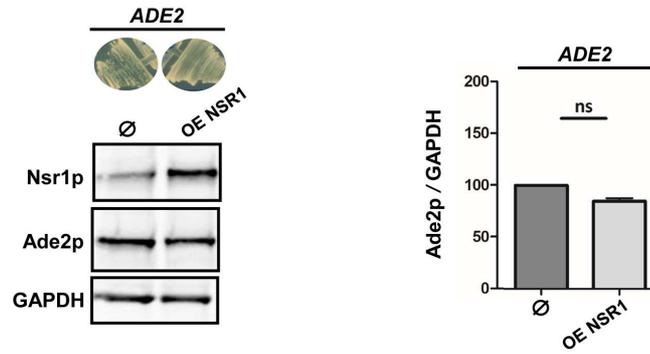


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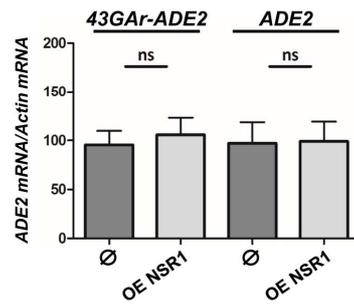


## Supplementary figure 1

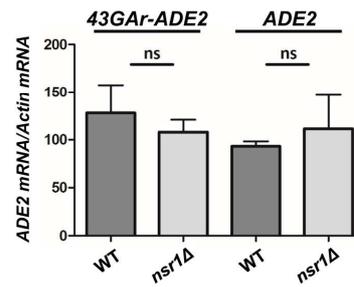
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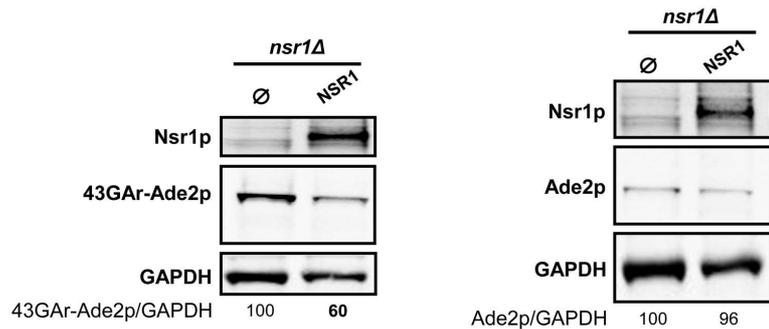
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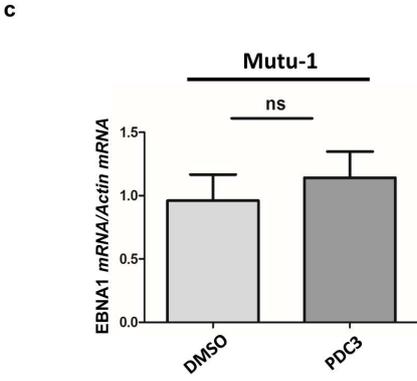
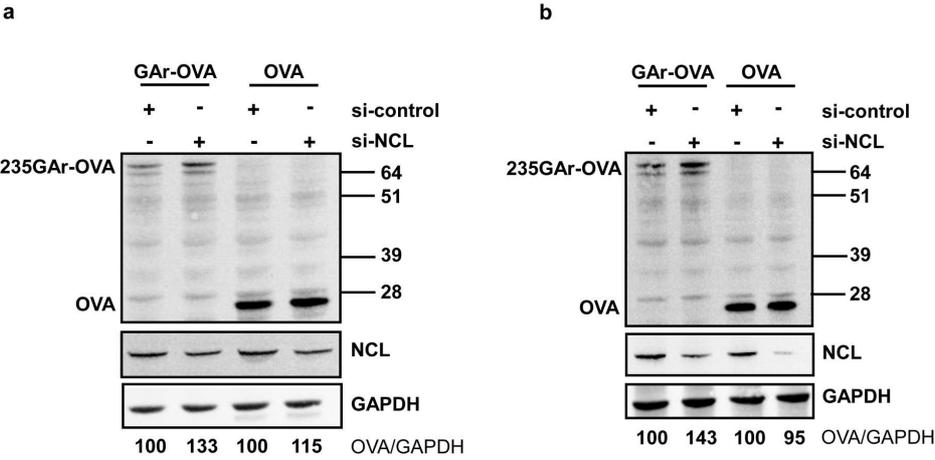
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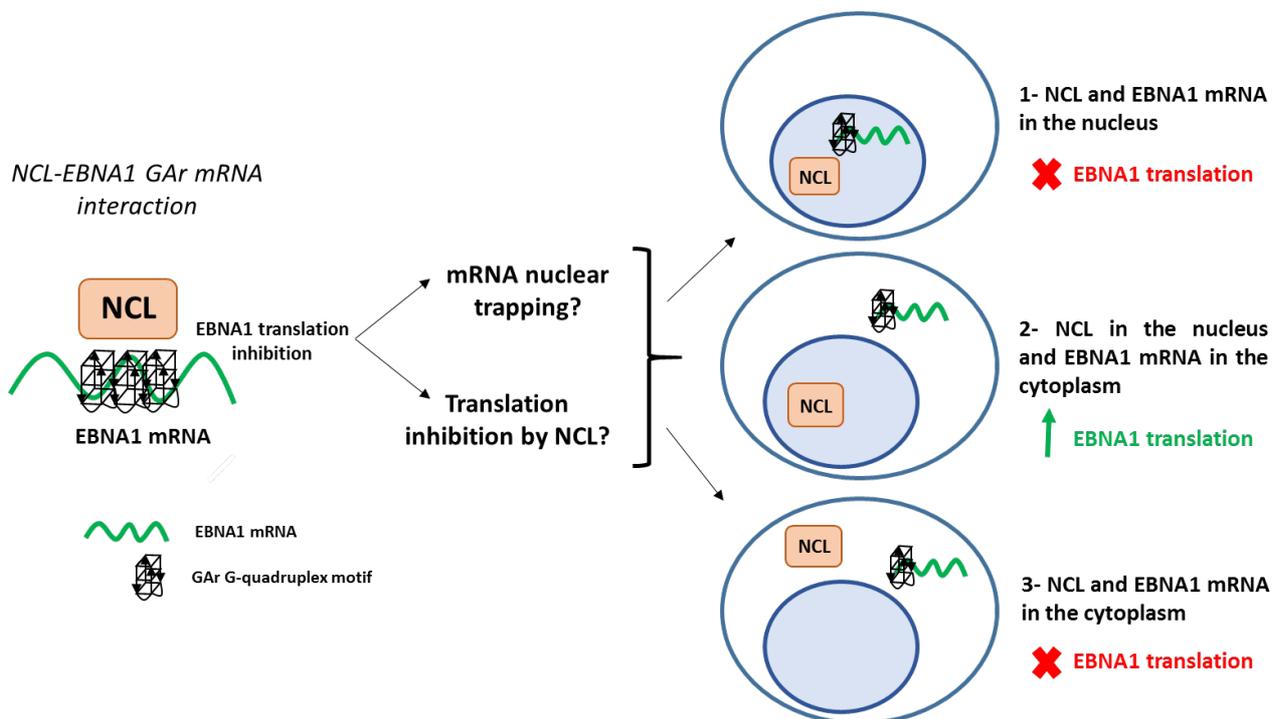
Supplementary figure 3



## Chapter 2:

Unraveling NCL involvement in the GAR-based translation inhibition: nuclear trapping, translation inhibition or both?

- **Graphical abstract:**



- ***Manuscript presentation:***

In the first part of this thesis we identified NCL as the first cellular factor able to interact directly with the GAR-encoding mRNA and affecting its translation inhibition effect. Furthermore, we determined that this interaction takes place in the nucleus and that a well-known G4 ligand (PhenDC3) is able to partially inhibit the binding of NCL to the G-quadruplex (G4) sequences of GAR mRNA and thereby prevent the GAR-based inhibition of translation and antigen presentation. We next wondered how this mechanism occurs. Is NCL trapping EBNA1 mRNA in the nucleus, thus explaining why the mRNA is poorly translated? Or does NCL itself inhibit translation through its binding to G4?

Our first question was to determine if the GAR translation inhibition is still effective when GAR-mRNA is mislocalized in the cytoplasm (intron-containing constructs or HIV-1 REV-based cytoplasmic transport). We demonstrated that these modifications of the localization of GAR mRNA from the nucleus to the cytoplasm abolishes its interaction with NCL as well as the GAR-based translation inhibition. We next showed that changing NCL localization from the nucleoplasm to the cytoplasm (by mutation of its nuclear localization signal) allows the interaction between the cytoplasmic GAR mRNA and the cytoplasmic NCL, and that this interaction reestablishes GAR-dependent inhibition of translation. These results indicate that NCL has a direct effect on the regulation of the GAR-based translation inhibition whatever the localization of its interaction with GAR mRNA. However, the fact that the nuclear localization of GAR mRNA correlates with the nuclear localization of NCL still suggests that nuclear trapping may also play a role. Hence, we have not formally determined yet if the GAR inhibition of translation involves both direct inhibition of translation and nuclear trapping, or if the nuclear trapping of GAR mRNA by NCL is solely responsible for GAR-based inhibition of translation.

These data were generated in collaboration with R. Prado-Martins in the lab of Dr. Robin Fåhræus. The manuscript presented below represents my interpretation of the results. These results will be part of a publication that is currently in preparation, from which I will be the second author.

## INTRODUCTION

The Epstein-Barr virus (EBV) is the first oncogenic virus described in human. EBV is worldwide spread causing a latent infection of B cells which is often asymptomatic. Nevertheless, EBV infection has been linked to several types of cancers like Burkitt and Hodgkin lymphomas, nasopharyngeal carcinoma and 10% of gastric cancers<sup>26</sup>. EBV's EBNA1 protein is the only protein expressed in all EBV-infected cells as it is essential for viral replication and maintenance. EBNA1 is also highly antigenic and T cells against EBNA1-derived antigenic peptides exist in all EBV-infected individuals. Therefore, EBV has evolved a strategy in order to tightly regulate EBNA1 protein level to ensure genome replication and maintenance and at the same time avoid T CD8<sup>+</sup> recognition of EBV-infected cells. EBNA1-based mechanism of immune evasion is based on its central glycine-alanine rich domain (GAR) which is able to self-limit the synthesis of its own mRNA *in cis*, and thus the production of EBNA1-derived antigenic peptides<sup>93, 90, 143</sup>. The mechanism involved in this GAR-based translation inhibition is still unknown and highly debated<sup>5, 92</sup>. Recent evidence has shown that the GAR-encoding mRNA form particular secondary structures known as G-quadruplexes (G4) which seem to be responsible for GAR-mediated inhibition of translation and antigen presentation<sup>98</sup>. Our group recently identified Nucleolin (NCL) as the first cellular factor critically involved in both GAR-based inhibition of translation and antigen presentation (Lista *et al* submitted, see chapter 1). We demonstrated that NCL directly binds to G4 formed by GAR-encoding mRNA and that this interaction takes place in the nucleus. Furthermore, impairing NCL binding to GAR mRNA by using PhenDC3 G4 ligand abolishes GAR-based inhibition of translation and antigenic presentation. Nevertheless, we still don't know how this interaction inhibits mRNA translation (Lista *et al* submitted, see chapter 1). Since NCL-GAR mRNA interaction takes place in the nucleus, we can hypothesize three mechanisms: first, GAR G4s may act as a recognition signal for NCL binding which would repress translation; second, NCL could trap GAR RNA in the nucleus which would explain its low translation level; third, a combination of the two hypotheses described above. Here we showed that loosing NCL-GAR RNA interaction completely abolishes GAR-based translation inhibition and also antigen presentation. Moreover, our data show that GAR-based translation inhibition and antigen presentation are effective as long as GAR RNA and NCL are allowed to interact, i.e: as long as NCL and GAR RNA are localized in the same cellular compartment.

Hence, the nuclear localization of the interaction between NCL and GAR RNA seems to be rather due to the cellular localization of NCL in the cell.

## MATERIALS and METHODS

### Plasmid constructions

Supplementary Table 1 corresponds to a list of plasmids, primers and restriction enzymes used in this study. We construct a set of reporters described in Figure 1. Briefly, full-length globin gene and OVA exons 5-7 fragment were amplified by PCR from the Glob-exon-SL8 construct<sup>144</sup> and chicken genomic DNA, respectively. The amplicons were cloned into pcDNA3 to create the control constructs (GG, OG). The OVA ORF of the pcDNA3-GAr-OVA construct<sup>90</sup> was replaced by the globin gene or OVA exons 5-7 fragment to generate plasmids in which the GAr domain is located upstream and in the same frame than the inserts (GGG, GOG). Afterwards, RNA from H1299 cells transfected with these gene constructs were used to generate cDNAs. The resultant cDNAs were used in a second round of cloning using the same primers and approach employed to produce the gene constructs to generate the cDNA constructs (GC, GGC, OC, and GOC). The GAr globin-RRE gene and GAr globin-RRE cDNA constructs were cloned according to the same method, using the plasmid Glob-exon-SL8-RRE<sup>144</sup> as template. The HA-NCLΔNLS plasmid was created by site-directed mutagenesis employing the pcDNA3-HA-NCL construct (Lista *et al*/submitted) as a template. The NLS sequence KKRA was replaced by AARA using the primers described in the Supplementary Table 1. The Rev expression vector was a gift from Ali Saïb, Saint-Louis Hospital, Paris. All constructs were amplified in DH5α *E. coli* and sequenced by Sanger method.

### Cell culture, transfection and drug treatments

The human cell line H1299 and the human HEK-293 T Kb stable cell line (kindly provided by L. Eisenlohr, Thomas Jefferson University, Philadelphia, PA) were cultured in RPMI-1640 and DMEM, respectively, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Transient transfections were performed using Genejuice reagent (Merck Bioscience) according to manufacturer's protocol. All cells were cultured at 37 °C with 5% CO<sub>2</sub>. For cell treatment with isoginkgetin (Sigma) and PhenDC3 (kindly provided by MP. Teulade-Fichou from Inserm U1196, Curie Institute, Orsay, France), cells were incubated with 30 µM and 5 µM of drug for 24h and 48h after transfection, respectively. Drug stock solutions were prepared in DMSO (Euromedex). All experiments, except for the antigen presentation assays, were carried out using H1299 cells.

### **RNA extraction, RT-PCR and RT-qPCR**

1, 5 x 10<sup>5</sup> H1299 cells were plated in six-well plates and transfected with the indicated constructs. At 48 h post-transfection, cells were washed with cold PBS 1X and RNA was purified using the RNeasy Mini Kit and on-column DNase treatment (Qiagen) following the manufacturer's protocol. Nuclear/cytoplasmic RNA extractions were carried out according to Apcher *et al*<sup>144</sup> employing transfected H1299 cells. RT was carried out using M-MLV reverse transcriptase and random hexamers (Invitrogen). q-PCR was performed using a set of primers targeting OVA or globin (see Supplementary Table 2) and Phusion high-fidelity PCR master mix (Thermo Scientific). RT-qPCR was performed as previously described<sup>144</sup> using specific primers for each gene fragment of interest (see Supplementary Table 2).

### **Western blot analysis**

Whole cell lysates were prepared 48 hours post-transfection and protein concentration was measured using a Bradford assay. Samples were electrophoretically separated in NuPAGE® 10% Bis-Tris gels (Invitrogen), transferred onto 0.45 µm nitrocellulose membranes (GE) and blotted under standard conditions using the following antibodies: mouse monoclonal anti-globin antibody (2H3, Sigma), rabbit polyclonal anti-OVA antibody (C6534 Sigma), mouse monoclonal anti-actin (AC-15 Sigma), mouse polyclonal anti-HA antibody (a kind gift from Borek Vojtesek, Masaryk Memorial Cancer Institute, Brno, Czech Republic) and chicken polyclonal anti-HIV1 Rev antibodies (ab36623, Abcam). Anti-mouse (Dako), anti-rabbit (Dako) or anti-chicken (Sigma) secondary antibodies conjugated to horseradish peroxidase were used according to manufacturer's recommendation to generate immunocomplexes revealed with enhanced chemiluminescence (Thermo Scientific). Membranes were scanned in a MyECL imager (Thermo Scientific) and signal intensity was determined using My Image software (Thermo Scientific).

### **Protein immuno-detection and RNA staining**

1 x 10<sup>4</sup> H1299 cells were plated on 12 mm-diameter coverslips in 24-well plates and transfected with the indicated constructs or empty pCDNA3. 24 h post-transfection, cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with PBS 1X 0.4% - Triton X-100 and 0.05% CHAPS for 10 minutes at room temperature and saturated with PBS 1X - 3% BSA for 30 minutes. Samples were incubated overnight at 4 °C with rabbit polyclonal anti-NCL (ab22758, Abcam) antibody or mouse

monoclonal anti-HA antibody conjugated to Alexa Fluor® 488 (6E2, Cell Signaling). Goat anti-rabbit Ig antibody conjugated to Alexa Fluor® 647 (Sigma) was used as secondary antibody when necessary. RNA-FISH assays were performed by using globin and OVA probes obtained from Biosearch Technologies according to manufacturer's protocol. For protein immune-detection coupled to RNA-FISH, samples were incubated with chicken polyclonal anti-HIV1 Rev antibody (ab36623, Abcam) after the FISH probes hybridization step and immunocomplexes were detected under standard conditions using goat anti-chicken IgY conjugated to Alexa Fluor® 568 (Abcam). Samples were examined in an LSM 800 confocal laser microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

#### **Proximity Ligation Assay (PLA) for RNA/protein interactions**

H1299 cells were grown, fixed and permeabilized as described above. Samples were hybridized overnight with 50 ng of a globin DNA probe (5'-GGCCTCACCACCAACTTCATCCACGTTACCTTGCAAAA-3') conjugated at digoxigenin in the 3' end. Afterwards, samples were saturated with the blocking solution (PBS 1X - 3% BSA - 0.1% saponine) and incubated for 2 hours with mouse monoclonal anti-digoxigenin (DI-22, Sigma) and rabbit polyclonal anti-NCL (ab22758, Abcam) primary antibodies diluted in blocking solution. The proximity ligation assay (PLA) was carried out using the Duolink PLA in situ kit (Sigma) according the manufacturer's protocol. For co-staining of HIV-Rev protein in PLA samples, chicken polyclonal anti-HIV1 Rev antibody (ab36623, Abcam) and goat anti-chicken IgY antibody conjugated to Alexa Fluor® 568 (Abcam) were added to primary antibodies and Duolink secondary antibodies mixes, respectively. For co-staining of NCLΔNLS, samples were incubated for 45 minutes with mouse monoclonal anti-HA antibody conjugated to Alexa Fluor® 488 (6E2, Cell Signaling) after the PLA amplification step.

#### **Antigen presentation assays**

Naive OVA<sub>257-264</sub> specific CD8<sup>+</sup> T cells were isolated by negative selection from peripheral and mesenteric lymph-nodes of 12-weeks-old female OT-1 mice using a CD8<sup>+</sup> T cell isolation kit (Miltenyi Biotec, Germany). Afterwards, CD8<sup>+</sup> T cells were stained with CellTrace™ Violet (Thermo Fisher Scientific, USA) according to the manufacturer's protocol and mixed with H1299 cells co-transfected with mouse Kb expression vector and the indicated constructs. For all the assays, 10<sup>5</sup> HEK-293 T Kb cells were harvested 48h after transfection and co-incubated with 4 x 10<sup>5</sup> stained CD8<sup>+</sup> T cells at 37 °C in humidified air/CO<sub>2</sub> atmosphere in RPMI medium containing 10% FBS, 4 mM L-glutamine, 100

4

U/ml penicillin, 100 µg/ml streptomycin, 5 mM HEPES and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich). After 3 days, cells were harvested, stained with hamster anti-mouse CD3-APC (Miltenyi Biotec) antibody and the fixable viability dye eFluor® 780 (eBioscience, USA) and analyzed by FACS on a CANTO II flow cytometer (BD Biosciences, USA). Cells were gated for live CD3<sup>+</sup> cells (10000 events collected) and data were analyzed using BD FACSDiva software version 8.0.1. The percentage of proliferating T cells was considered for statistical analysis. For IL-2 production analysis, supernatants were collected after 3 days of co-incubation and IL-2 levels were measured employing the IL-2 ELISA MAX™ Standard kit (Biolegend, USA) according to manufacturer's instructions.

#### **Statistical analysis**

Data were analyzed by ANOVA in conjunction with Tukey's test or two-tailed unpaired Student's t-test using GraphPad Prism 5 for Windows. Data shown are mean ± s.d. of a minimum of three independent experiments. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; ns, not significant.

## **RESULTS and DISCUSSION**

### **Interaction of Nucleolin with GAR-carrying RNAs only takes place in the nucleus**

We recently demonstrated that GAR mRNA-Nucleolin (NCL) interaction takes place in the nucleus and that this interaction is essential to ensure GAR-based translation inhibition (Lista *et al*, submitted). We first wondered if EBNA1 mRNA translation inhibition may be due to its trapping by NCL in the nucleus, thus avoiding EBNA1 mRNA cytoplasmic transport and translation. For this purpose, we took advantage of the fact that pre-RNAs (intron-containing RNA) that need to be spliced are taken over by the splicing machinery which rapidly mediates the transport of spliced-mRNAs into the cytoplasm for their subsequent translation. For this reason, we created several constructs containing the GAR-encoding sequence in fusion with globin or ovalbumin cDNA (GGC and GOC) or complete gene sequence (GGG and GOG), as well as the GAR-free corresponding controls (GC,GG,OC,OG) (**Figure 1A**). Each construct contains the ovalbumin (OVA) SL8 antigenic peptide SIINFKEL (OVA<sub>257-264</sub>) sequence in order to measure the antigenic presentation of the peptides derived from all construct used. By fluorescence *in situ* hybridization (FISH) and subcellular fractionation, we showed that, as expected, both GAR-free RNAs (GG and OG) and GAR-carrying RNAs (GGG and GOG) from the gene constructs are localized in the cytoplasm (**Figure 1B-C**). However, GAR-carrying RNAs from cDNA

constructs (GGC and GOC) are localized in the nucleus (**Figure 1B-E**), contrary to GAR-free RNAs from cDNA constructs (GC and OC). We then checked the ability of GAR-carrying RNAs localized in the nucleus or the cytoplasm to interact with NCL by proximity ligation assay (PLA) and RNA co-immunoprecipitation. We demonstrated that only GAR RNAs localized in the nucleus are able to specifically interact with NCL whereas no interaction was detected between NCL and GAR cytoplasmic RNAs (**Figure 2A-B**). To support our findings, we treated cells expressing GAR cytoplasmic RNAs with the splicing inhibitor isoginkgetin. As expected, this treatment inhibited the cytoplasmic transport of GAR RNAs from gene constructs (GGG) which were therefore located in the nucleus. Using PLA, we demonstrated that these GAR RNAs from gene constructs (GGG) which are held back in the nucleus, are able to establish an interaction with NCL (**Figure 2C**). Taken together our results showed that in order to be able to interact with NCL, GAR-carrying RNAs need to be located in the nucleus, which is in good agreement with the location of NCL in the cell.

#### **GAR-based inhibition of translation and antigen presentation is solely efficient from nuclear RNAs.**

After having demonstrated that only GAR RNAs present in the nucleus are able to interact with NCL, we tested the translation efficiency from nuclear and cytoplasmic GAR-carrying RNAs. For this purpose, H1299 cells were transfected with the globin (**Figure 3A**) and OVA (**Figure 3D**) constructs, and their expression levels were analyzed by western blot. We showed that GAR is solely able to inhibit the expression of nuclear RNAs (GGC, GOC) whereas its effect is completely abolished when the RNAs are located in the cytoplasm (GGG, GOG; **Figure 3A and D**). Because GAR-based translation inhibition affects the production of antigenic peptides and thus the recognition of infected cells by specific T CD8<sup>+</sup> cells, we next measured the effect of GAR RNA localization on T CD8<sup>+</sup> cells proliferation. For this purpose, we determined the proliferation rate of naïve T CD8<sup>+</sup> cells (OT1 cells) recognizing specifically the OVA<sub>257-264</sub> SIINFEKL epitope complexed to MHC class I molecule. The OT1 cells were first isolated from peripheral and mesenteric lymph-nodes of 12-weeks-old mice and stained with the CellTrace™ Violet fluorescent dye. OT1 cells were then mixed with HEK-293 T Kb expressing nuclear or cytoplasmic GAR-globin and GAR-OVA RNAs. As controls, cells expressing globin and OVA RNAs were used. As expected, antigenic peptides derived from GAR nuclear RNAs (GGC and GOC) are poorly detected by T cells (3,87% ± 1,96; p=0,0055\*\* for GGC and 19,80% ± 3,80; p=0,0002\*\*\* for GOC) whereas cytoplasmic GAR RNAs (GGG and GOG)-derived antigenic peptides lead to a strong activation of T CD8<sup>+</sup> cells (70,17% ± 17,11; p=0,9204 ns for GGG and 83,97% ± 4,73;

p=0,1496 ns for GOG) (**Figure 3B-C and E-F**), similarly as what is observed for GAR free globin controls (GG, GC, OG and OC). These results showed that NCL interaction is essential to ensure the GAR-based inhibition of translation and antigenic presentation.

**Export of nuclear GAR RNAs to the cytoplasm abolishes GAR RNA-NCL interaction and suppresses the GAR-based inhibition of translation and antigen presentation.**

We next wondered if the relocation of nuclear GAR RNAs in the cytoplasm may affect its interaction with NCL, GAR-fused protein expression and antigenic presentation.

In order to modify the localization of nuclear GAR RNAs we inserted the RRE recognition sequence for HIV-1 REV protein in the globin cDNA constructs (**Figure 4A-B**). During HIV-1 infection some un-spliced viral mRNAs coding for structural proteins need to be transported to the cytoplasm to be rapidly expressed. To do so, the REV viral protein is expressed in the early stage of HIV-1 infection and promotes the cytoplasmic transport of RRE-mRNAs. By using this REV-based system we showed that nuclear GAR-globin RNAs (GGC) are efficiently exported to the cytoplasm by REV (**Figure 4B**). The interaction between GAR RNA and NCL, that is taking place in the nucleus (**Figure 4C**), is completely abolished after RNA transport to the cytoplasm (**Figure 4D**). Furthermore, the cytoplasmic transport of GAR RNA from cDNA construct (GGC) enhances GAR globin expression (**Figure 4E**) and also antigen presentation (**Figure 4G**). Altogether these data, demonstrate that NCL co-localizing with GAR RNA in the nucleus is crucial to ensure their interaction and a further GAR-based translation inhibition effect.

**NCL-mediated GAR inhibition of translation is also efficient in the cytoplasm.**

We finally sought to determine whether the nuclear location of NCL-GAR RNA interaction is essential to ensure the GAR inhibition of translation effect. For that purpose, we constructed a cytoplasmic NCL mutant by modifying its nuclear localization signal (NCL $\Delta$ NLS, **Figure 5A**). We first showed by PLA that changing NCL location from the nucleus to the cytoplasm restores its interaction with cytoplasmic GAR RNAs GGG (**Figure 5B-C**). These data were confirmed by using the REV-based relocation of GAR RNAs from cDNA constructs in the cytoplasm: NCL $\Delta$ NLS interacts with cytoplasmic GAR RNAs from cDNA constructs GGC (**Figure 5F-G**). These data proves that NCL-GAR RNA interaction can take place outside of the nucleus. Moreover, we observed that the cytoplasmic interaction of NCL with GAR RNA restores the GAR-based translation inhibition (**Figure 5D-E**), demonstrating that to ensure EBNA1 translation inhibition, the interaction of GAR-containing RNA with NCL is essential no matter where it takes place in the cells (**Figure 5H**). Nevertheless, our results do not allow to

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determine if NCL inhibits translation of EBNA1 mRNA by a direct binding or if EBNA1 mRNA trapping in the nucleus by itself is sufficient enough for the inhibition of EBNA1 translation and antigenic presentation. However, the fact that GAR mRNA-NCL cytoplasmic interaction also leads to translation inhibition indicates that NCL can inhibit protein translation without RNA nuclear trapping. Moreover, we can hypothesize that NCL interaction with GAR RNA may block the binding of the ribosomal subunit 40S, the ribosomal assembly of the subunit 60S or the ribosomal progression. Another possibility is that GAR polypeptide may also be involved in the translation inhibition by blocking ribosomal read-through.

Taken together our results demonstrated that in order to ensure EBNA1 GAR-based translation inhibition, NCL is recruited to the EBNA1 mRNA through its interaction with GAR G4s motif which inhibits EBNA1 translation and thus antigen presentation.

#### FIGURE LEGENDS

**Figure 1** – The nuclear or cytoplasmic localization of RNA is driven by the presence of the GAR domain.

(A) Scheme illustrating the constructs employed in this study. A set of globin and chicken ovalbumin (OVA) constructs was generated with (GGG, GGC, GOG, GOC) or without (GG, GC, OG, OC) the GAR sequence in 5' of open reading frames. The gene-based constructs contain introns (GGG, GOG, GG and OG) or not (cDNA-based constructs GGC, GOC, GC and OC). SL8 denotes the OVA-derived SIINFEKL antigenic peptide inserted into the exon 1 of the globin constructs. SL8 peptide is naturally present in OVA-derived constructs.

(B) FISH staining of globin RNA. Fusion of GAR to the globin cDNA shifts the localization of the RNA from predominantly cytoplasmic (GC) to largely nuclear (GGC). In contrast, GAR has no effect on the localization of the spliced RNA (GG and GGG). Red and blue staining represent RNA and DAPI, respectively. Scale bar = 10  $\mu$ m.

(C) Cells transfected with the indicated constructs were fractionated for analysis of RNA content of the nucleus and cytoplasm by RT-qPCR. The ratio of nuclear to cytoplasmic RNA was calculated showing a relative nuclear accumulation of intronless GAR-Globin RNA (GGC). Histogram bars represent the mean of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , ns: not significant.

(D) FISH staining of ovalbumin (OVA) RNA. Fusion of GAR to the OVA cDNA shifts the localization of the RNA from predominantly cytoplasmic (OC) to largely nuclear (GOC). In contrast, GAR has no effect on the localization of the spliced RNA (OG and GOG). Red and blue staining represent RNA and DAPI, respectively. Scale bar = 10  $\mu$ m.

(E) Cells transfected with the indicated constructs were fractionated for analysis of RNA content of the nucleus and cytoplasm by RT-qPCR. The ratio of nuclear to cytoplasmic RNA was calculated showing a relative nuclear accumulation of intronless GAR-OVA RNA (GOC). Histogram bars represent the mean of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , ns: not significant.

**Figure 2** – Only nuclear GAR-carrying RNAs are able to interact with nucleolin.

(A) Proximity ligation assays (PLA) carried out between nucleolin (NCL) and globin RNA in cells transfected with globin (GG and GC) or GAR-globin (GGG and GGC) constructs. NCL-globin RNA PLA complexes correspond to red dots and each dot represents an interaction between nucleolin and globin RNA. Nucleolin interacts exclusively with the globin RNA in the context of GAR RNA (GGC) from cDNA constructs (*lower right*), predominantly in the nucleus (*white arrows*). No interactions are observed from the intron-containing GAR-Globin (GGG) or control (GG and GC) constructs. Blue staining represents DAPI. White and red scale bars denote 20 and 10  $\mu$ m, respectively.

(B) Cells transfected with indicated constructs were subjected to RNA co-immunoprecipitation (RNA-coIP) using antibodies against nucleolin. NCL and IgG bound RNAs were analyzed by RT-qPCR against globin. This confirms the interaction of NCL with GAR-globin RNA from cDNA construct. Graphs represent the mean of three independent experiments. \*\* $p < 0.01$ , ns: not significant.

(C) Cells transfected with GAR-globin gene construct (GGG) were treated with 30  $\mu$ M of the spliceosome inhibitor isoginkgetin and subjected to proximity ligation assays (PLA) between NCL and the GGG RNA. Isoginkgetin treatment induces the retention of GGG RNA in the nucleus and the interaction between NCL and GGG RNA.

(D) Cells transfected with GAR-globin gene (GGG) and treated with 30  $\mu$ M of isoginkgetin were analyzed by RT-qPCR using primers located in introns, revealing that isoginkgetin treatment induces an accumulation of unspliced GGG RNA.

**Figure 3** – GAR suppresses protein synthesis and MHC class I-restricted antigen presentation only from GAR-carrying RNAs present in the nucleus.

(A) Western blots revealing that the GAR-mediated translation inhibition is not effective from cytoplasmic RNAs. One representative experiment of globin constructs is shown. Graphs show the mean of globin/actin proteins from three independent experiments. \*\*\* $p < 0.001$ .

(B) T cell proliferation assays. Human HEK-K<sup>b</sup> cells expressing globin constructs containing the SIINFEKL epitope (see **Figure 1A**) were co-incubated with CD8<sup>+</sup> T cells from OT-1 mice labelled with Cell-trace Violet. After three days, OT-1 cells were analyzed by FACS. The open peaks of the histogram represent successive generations of CD8<sup>+</sup> T cells indicating T cells activation. Unstimulated control cells are indicated in grey. Similar levels of antigen presentation are observed for controls (GG and GC) and GAR-globin gene construct (GGG). Expression of GAR-Globin cDNA construct (GGC) failed to stimulate OT-1 cells.

(C) Graph depicting the mean percentage of dividing CD8<sup>+</sup> T cells from three independent experiments. \*\* $p < 0.01$ , ns: not significant.

(D) Western blots revealing that the GAR-mediated translation inhibition is disrupted from cytoplasmic RNAs. One representative experiment of OVA constructs is shown. Graphs show the mean of OVA/actin proteins from three independent experiments. \*\*\* $p < 0.001$ .

(E) T cell proliferation assays. Human HEK-K<sup>b</sup> cells expressing OVA constructs containing the SIINFEKL epitope (see **Figure 1A**) were co-incubated with CD8<sup>+</sup> T cells from OT-1 mice labelled with Cell-trace Violet. After three days, OT-1 cells were analyzed by FACS. The open peaks of the histogram represent successive generations of CD8<sup>+</sup> T cells indicating T cell activation. Unstimulated control cells are indicated in grey. Similar levels of antigen presentation are observed for controls (OG and OC) and GAR-OVA gene construct (GOG). Expression of GAR-OVA cDNA construct (GOC) failed to stimulate OT-1 cells.

(F) Graph depicting the mean percentage of dividing CD8<sup>+</sup> T cells from three independent experiments. \*\* $p < 0.01$ , ns: not significant.

**Figure 4** – Changing GAR RNA location from nucleus to cytoplasm prevents its interaction with nucleolin and disrupts the capacity of GAR to suppress RNA translation and antigen presentation.

(A-B) FISH staining of GGC globin RNA and immunofluorescence detection of HIV-1 Rev. The Rev response element (RRE) sequence was fused to the 3'-UTR of GAR-globin cDNA construct to generate the GAR-globin-RRE cDNA construct (GGC-RRE). The RNA expressed from GGC-RRE is predominantly nuclear (A). In the presence of Rev, GGC-RRE RNA is exported to the cytoplasm in a Rev-dependent fashion (B).

(C-D) Proximity ligation assays (PLA) carried out between GGC RNA and NCL. Nucleolin interacts with GGC-RRE RNA in the nucleus (C). This interaction is impeded upon nuclear export of GGC-RRE RNA (D). PLA complexes are depicted as light blue dots and each dot represents an interaction between NCL and globin RNA. In A-D, dark blue staining represents DAPI. Scale bar = 10  $\mu$ m.

(E) Western blot analysis of cells transfected with Rev expressing vector or empty pcDNA3 in combination with the RRE sequence fused to GAR-globin cDNA construct (GGC-RRE). Nuclear-export of GGC-RRE RNA by Rev interferes with the GAR-mediated inhibition of translation, resulting in an increased level of globin expression.

(F) Graph showing the fold change of globin/actin levels relative to controls. This represents three independent experiments. \*\*p<0.01.

(G) HEK-Kb cells were co-transfected with Rev expressing vector or empty pcDNA3 and GGC-RRE construct. After three days of co-incubation with SL8 specific CD8<sup>+</sup> T cells from OT-1 mice, T cells supernatants were harvested and analyzed for IL2 levels. Nuclear-export of GGC-RRE RNA by Rev produces an increase of antigen presentation. Graph represents three independent experiments. \*\*p<0.01.

**Figure 5-** Interaction of nucleolin with GAR-carrying RNAs is sufficient to suppress RNA translation and antigen presentation.

(A) Immunofluorescence shows that endogenous NCL is predominantly localized in the nucleus (*left*). Cells were transfected with a HA-tagged NCL construct lacking the nuclear localization signal (HA-NCL $\Delta$ NLS) and immunofluorescence carried out using an anti-HA antibody (*right*) shows that NCL $\Delta$ NLS is predominantly localized in the cytoplasm. NCL staining is depicted in green. Dark blue staining represents DAPI. Scale bar = 10  $\mu$ m.

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(B-C) Proximity ligation assays (PLA) carried out between NCL $\Delta$ NLS and GGG RNA using anti-HA antibodies and a globin probe. No interaction is observed between endogenous NCL and GAR-globin gene (GGG) RNA (B). However, this interaction is observed upon the expression of HA-NCL $\Delta$ NLS in the cytoplasm (C). PLA complexes are depicted as light blue dots and each dot represents an interaction between NCL and globin RNA. Dark blue staining represents DAPI. Scale bar = 10  $\mu$ m.

(D) Western blot shows that HA-NCL $\Delta$ NLS prevents the protein synthesis from of cytoplasmic GAR-globin (GGG) RNA from the gene construct but not from the nuclear GAR-globin RNA from the cDNA construct (GGC). (E) Graph showing the fold change of globin/actin protein levels relative to controls and representing three independent experiments. \*\*\*p<0.001, ns: not significant.

(F) Expression of HA-NCL $\Delta$ NLS does not interfere with the interaction between GAR-globin-RRE RNA from the cDNA construct (GGC-RRE) and endogenous NCL in the nucleus (G) PLA carried out between NCL $\Delta$ NLS and GGC-RRE RNA using HA antibody and a globin probe shows that nuclear export of GGC-RRE RNA by REV interacts with HA-NCL $\Delta$ NLS in the cytoplasm. PLA complexes are depicted as light blue dots and each dot represents an interaction between NCL and globin RNA. Dark blue staining represents DAPI. Scale bar = 10  $\mu$ m.

(H) Schema summarizing the data obtained in panels A-G.

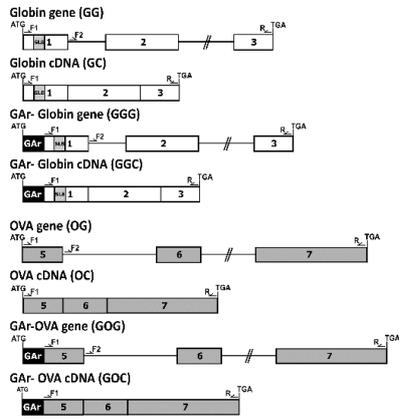
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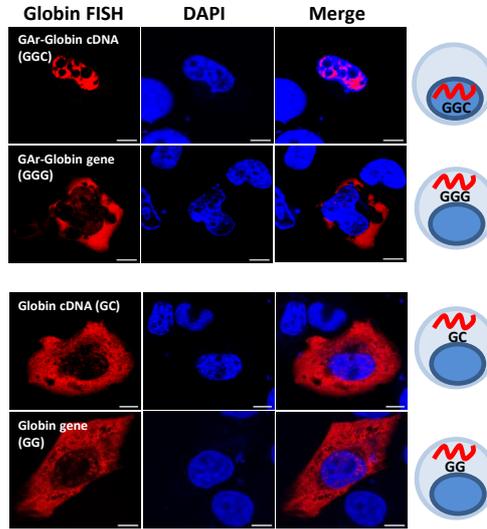
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**Figure 1**

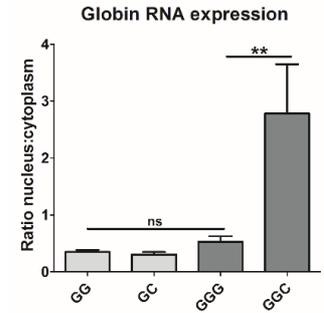
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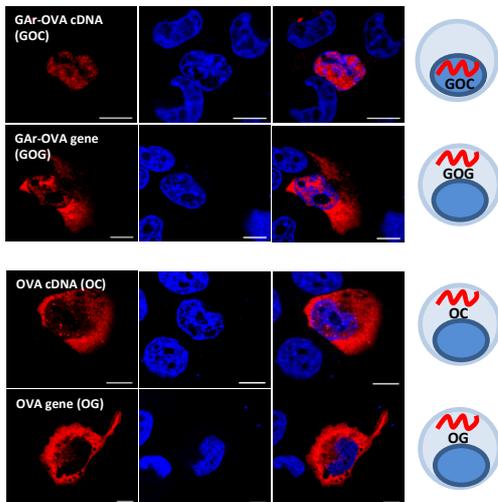
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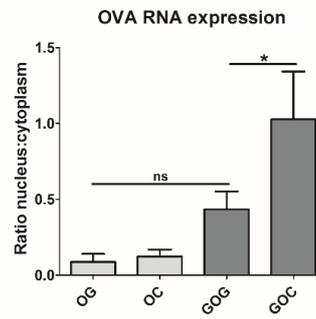
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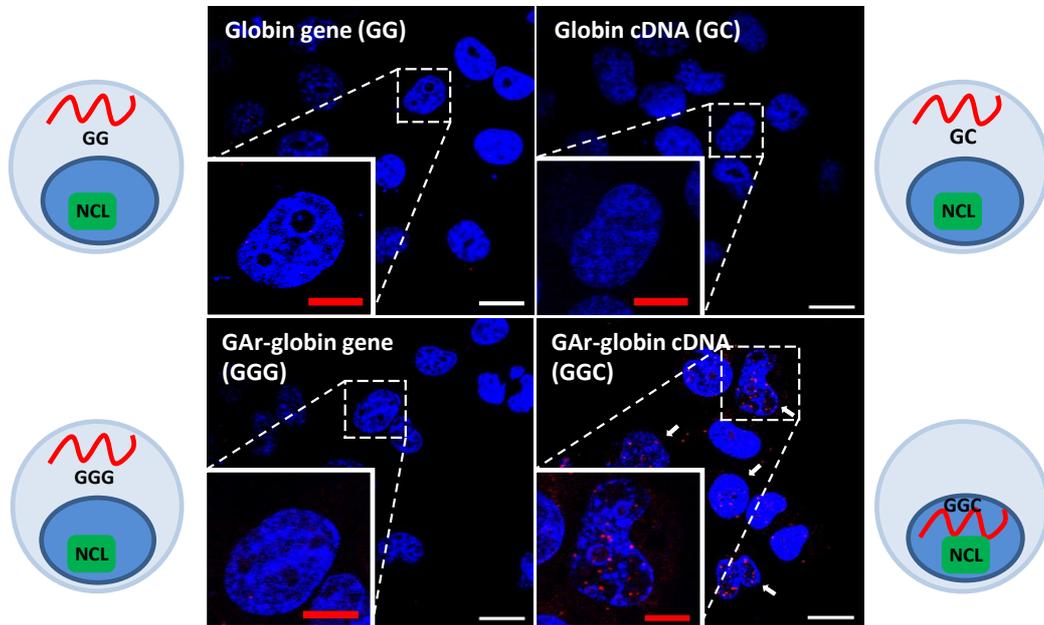


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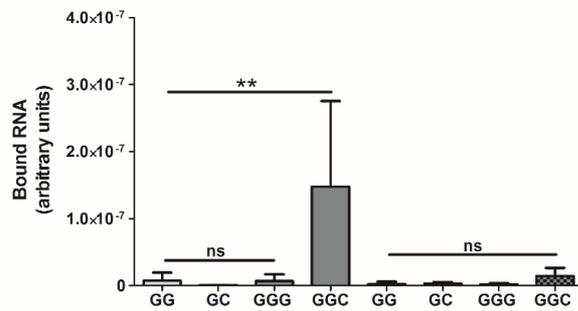
**Figure 2**

**A**

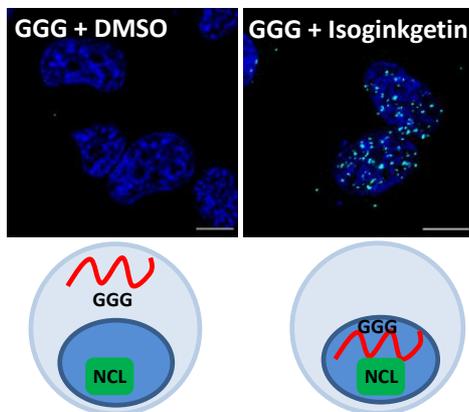


**B**

*in vivo* RNA-nucleolin co-IP

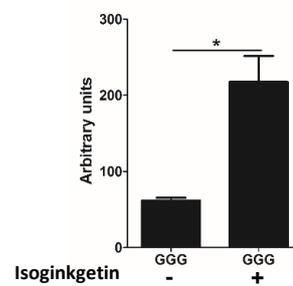


**C**

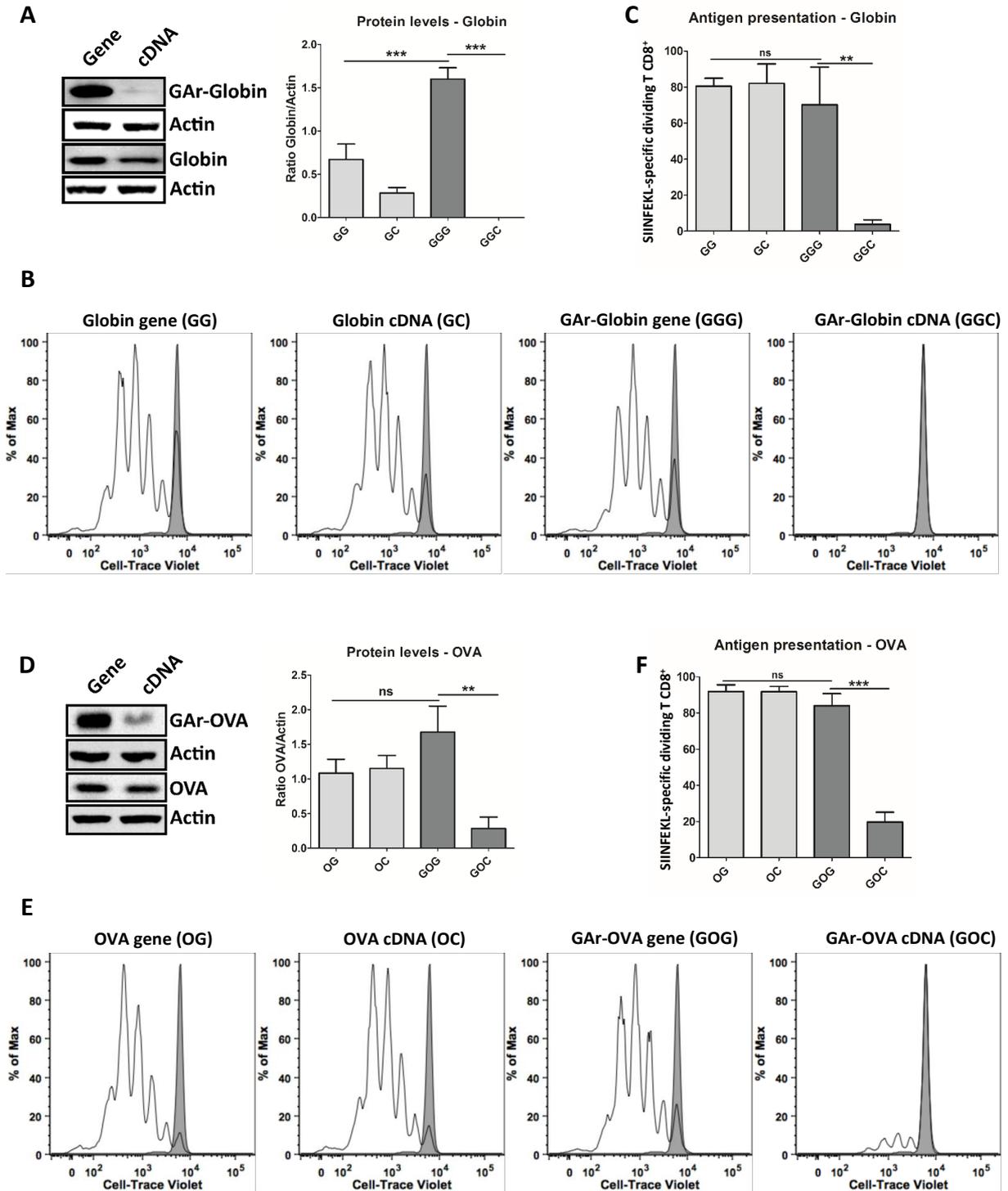


**D**

Globin pre-RNA expression



**Figure 3**



**Figure 4**

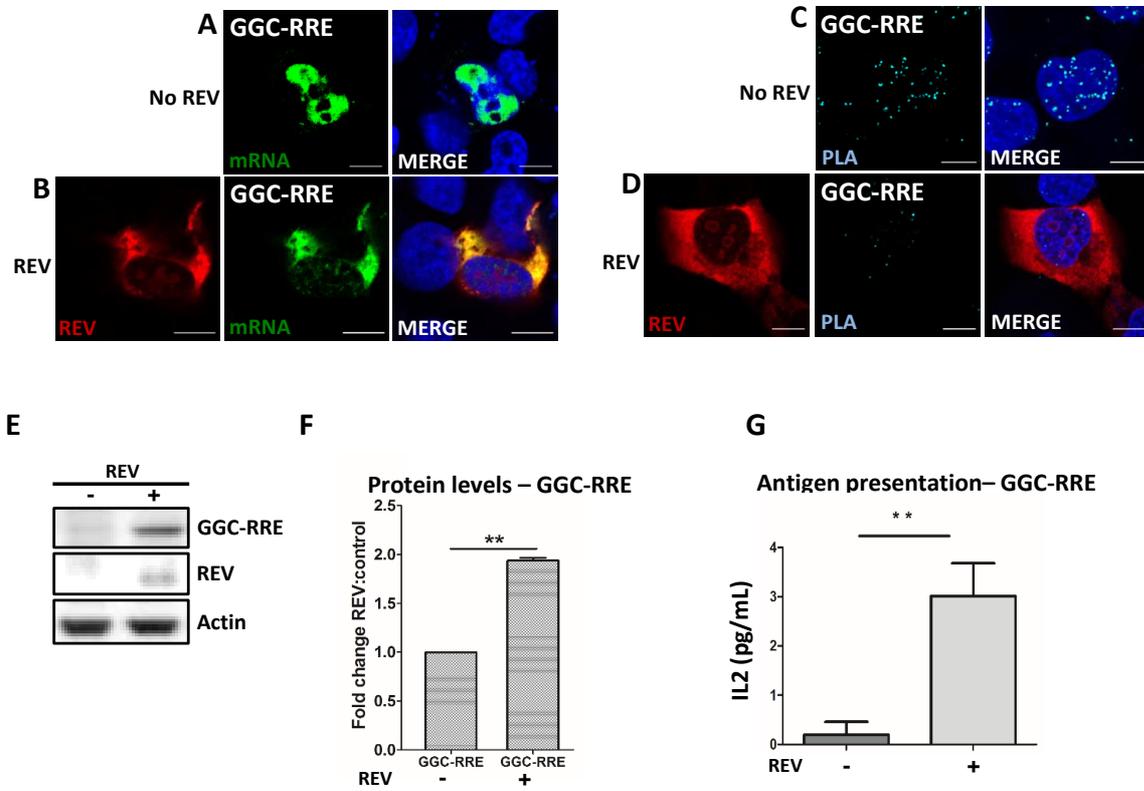
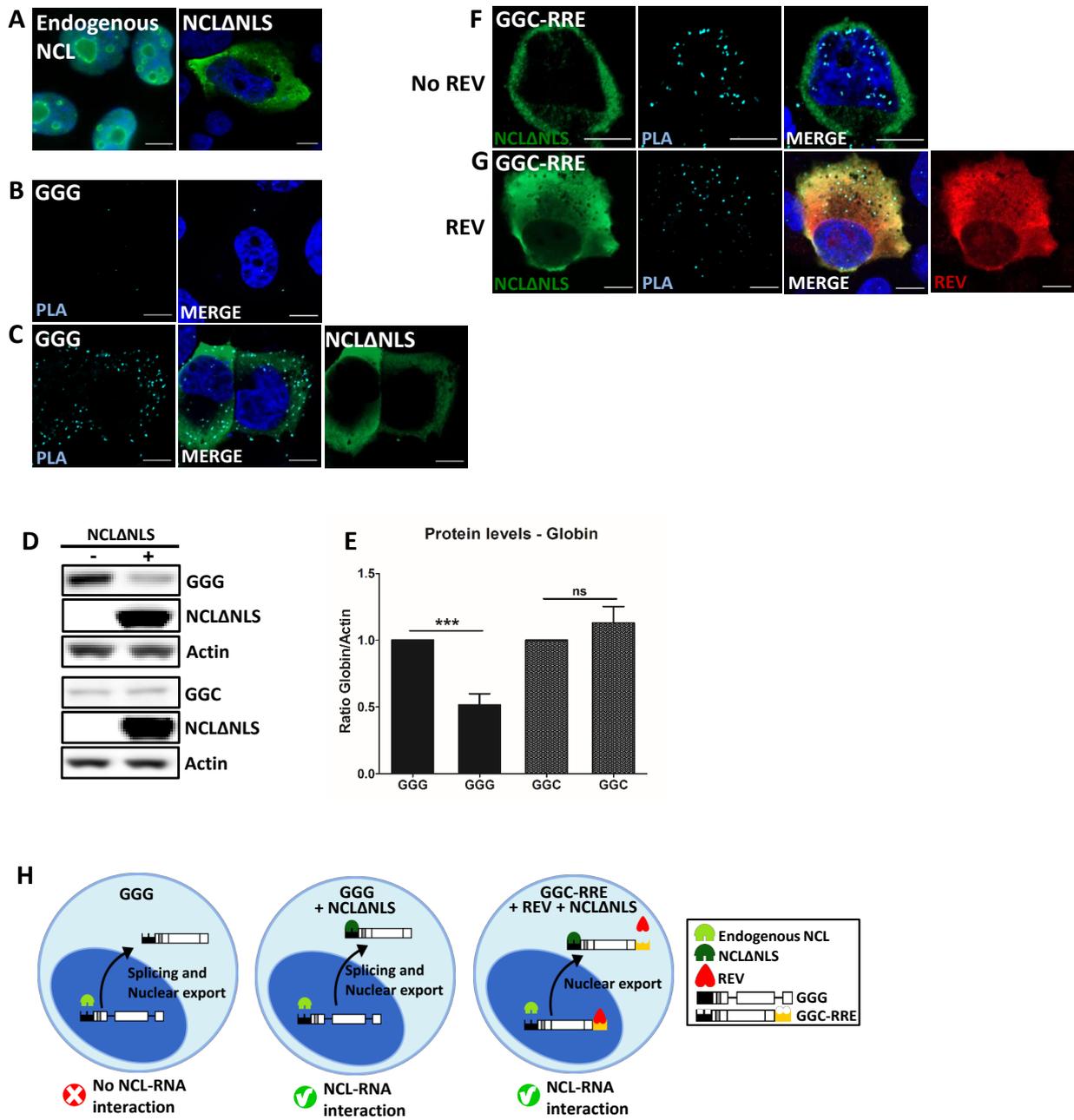


Figure 5



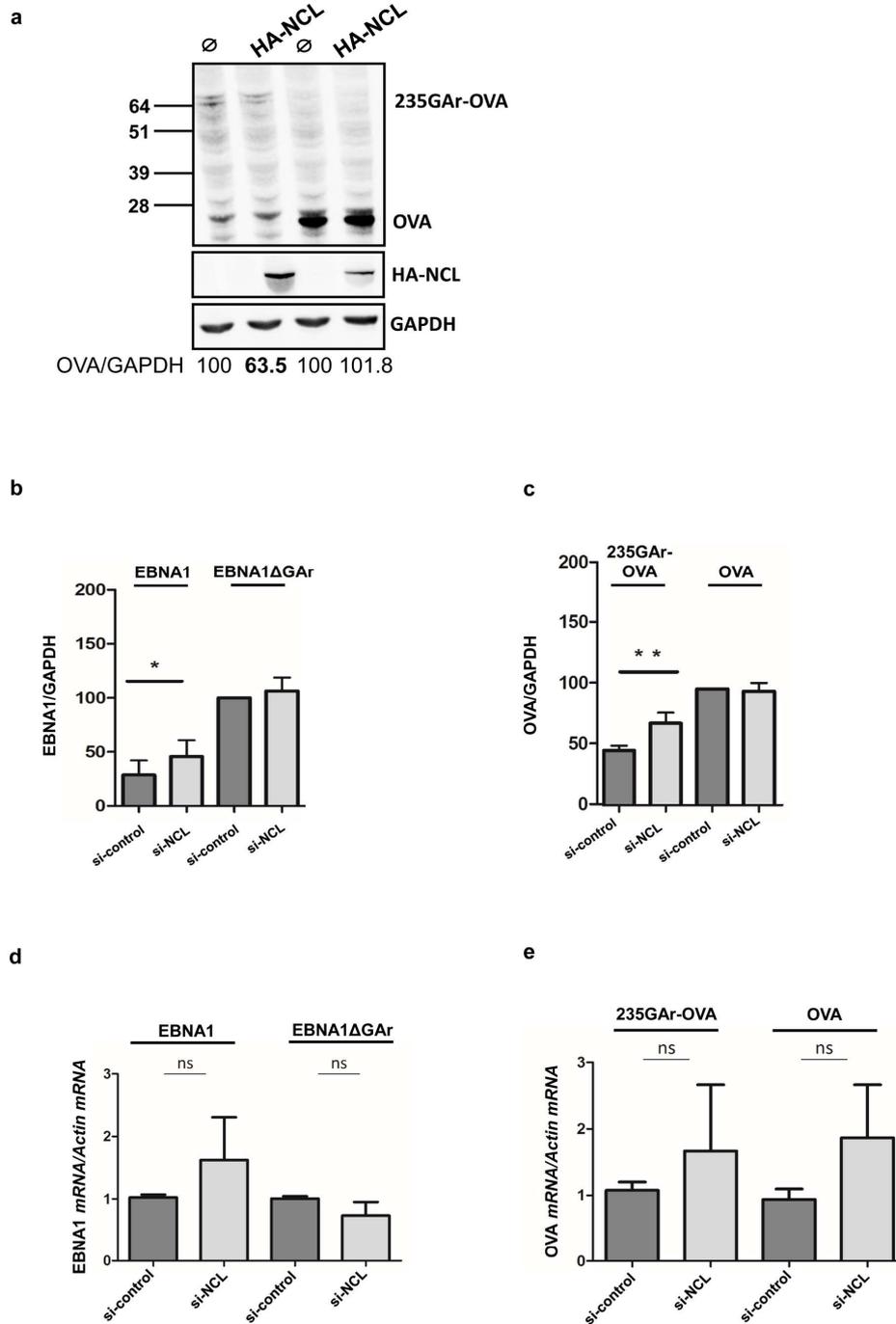
Supplementary Table 1 – Plasmids, primers and restriction enzymes used in this study.

| Constructs         | Forward primer (5'-3')   | Reverse (5'-3')  | Restriction enzymes |
|--------------------|--|--|---------------------|
| <i>Globin</i>      |  |  |                     |
| <b>GGG</b>         | GCGAATTGTGCACCTGACTCCTGA                                       | TAGGGCCCTTACTGGAAAGGCAGCCTG                              | EcoRI / ApaI        |
| <b>GGC</b>         | GCGAATTGTGCACCTGACTCCTGA                                       | TAGGGCCCTTACTGGAAAGGCAGCCTG                              | EcoRI / ApaI        |
| <b>GG</b>          | TTGAATTGTGATGTGCACCTGACTCCTGA                                  | TAGGGCCCTTACTGGAAAGGCAGCCTG                              | EcoRI / ApaI        |
| <b>GC</b>          | TTGAATTGTGATGTGCACCTGACTCCTGA                                  | TAGGGCCCTTACTGGAAAGGCAGCCTG                              | EcoRI / ApaI        |
| <i>Globin-RRE</i>  |  |  |                     |
| <b>GGGR</b>        | GCGAATTGTGCACCTGACTCCTGA                                       | TTGGGCCCTTAGATACTCCAATAAGCA                              | EcoRI / ApaI        |
| <b>GGRR</b>        | GCGAATTGTGCACCTGACTCCTGA                                       | TTGGGCCCTTAGATACTCCAATAAGCA                              | EcoRI / ApaI        |
| <i>Ovalbumin</i>   |  |  |                     |
| <b>OG</b>          | GCGCCTCGAAGATGGGAATTCAGAAATGTCCTTCAGCC                         | TTAGGGCCCTTAAAGGGGAAAACATCTGCC                           | XhoI / ApaI         |
| <b>OC</b>          | GCGCCTCGAAGATGGGAATTCAGAAATGTCCTTCAGCC                         | TTAGGGCCCTTAAAGGGGAAAACATCTGCC                           | XhoI / ApaI         |
| <b>GOG</b>         | GCGCCTCGAAGGAATTATCAGAAATGTCCTTCAGCC                           | TTAGGGCCCTTAAAGGGGAAAACATCTGCC                           | XhoI / ApaI         |
| <b>GOC</b>         | GCGCCTCGAAGGAATTATCAGAAATGTCCTTCAGCC                           | TTAGGGCCCTTAAAGGGGAAAACATCTGCC                           | XhoI / ApaI         |
| <i>Nucleolin</i>   |  |  |                     |
| <b>HA-NCLEANLS</b> | AAACAGAAAACAGCTCTCTGAAAGCCGCGCACAGGCAAGTGGAAAGCACAGAAACCGACTAC | GTAGTCGGTCTGTGCTTCCACTGCTGTGCGCGGCTTACGGAAGCTGCTTTCTGTTT | .....               |

Supplementary Table 2 – Primers used for RT-PCR and RT-qPCR

| <b>Primer</b>             | <b>Sequence (5'-3')</b>       |
|---------------------------|-------------------------------|
| <b>Figure 1</b>           |                               |
| Globin F1                 | ATGGTGACCTGACTCCTGA           |
| Globin F2                 | TATCAAGGTTACAAGACAGG          |
| Globin R                  | CTGGAAGGCAGCCTG               |
| OVA F1                    | GGAATTATCAGAAATGTCCTTCAGCC    |
| OVA F2                    | GGGCATACCTTAGAGATGTAATCTAG    |
| OVA R                     | TTAAGGGGAAACACATCTGCC         |
| <b>Figures 3, 4 and 7</b> |                               |
| Globin exon 2 FW          | CTGCTGGTGGTCTACCCTTG          |
| Globin exon 2 RV          | AGCTTGTCACAGTGCAGCTC          |
| Globin intron 1 FW        | TATCAAGGTTACAAGACAGGTTTAAGGAG |
| Globin intron 1 RV        | ACCACCAGCAGCCTAAGGGTG         |
| <b>Figure 3</b>           |                               |
| OVA exon 6 FW             | GCAAACCTGTGCAGATGATG          |
| OVA exon 6 RV             | CTGCTCAAGGCCTGAGACTT          |

## Supplementary figure 2



## Chapter 3:

### Yeast as a tool to decipher some of the steps of EBV life cycle

- ***Manuscript presentation:***

During the past 30 years the yeast *Saccharomyces cerevisiae* happens to have been an excellent tool to study some of the steps of the Epstein-Barr virus life cycle. From the beginning of the eighties yeast has been used in order to identify the autonomous replicative sequences (ARS) of EBV genome, and also to study EBV lytic cycle. Later on, yeast has contributed to the study of EBNA1 function in the maintenance of the viral episome. Moreover, thanks to the yeast two-hybrid assay the cellular proteins that collaborate with EBNA1 in order to ensure the episome maintenance were identified. Finally, yeast served to explore EBNA1 GAR-based mechanism of immune evasion. These topics were the subject of a review that we recently published in Biotechnology Journal which manuscript is presented below.

Review

## The long-lasting love affair between the budding yeast *Saccharomyces cerevisiae* and the Epstein-Barr virus

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The Epstein-Barr gammaherpesvirus (EBV) is the first oncogenic virus discovered in human. Indeed, EBV has been known for more than 50 years to be tightly associated with certain human cancers. As such, EBV has been the subject of extensive studies aiming at deciphering various aspects of its biological cycle, ranging from the regulation of its genome replication and maintenance to the induction of its lytic cycle, including the mechanisms that allow its immune evasion or that are related to its tumorigenicity. For more than 30 years the budding yeast *Saccharomyces cerevisiae* has fruitfully contributed to a number of these studies. The aim of this article is to review the various aspects of EBV biology for which yeast has been instrumental, and to propose new possible applications for these yeast-based assays, as well as the creation of further yeast models dedicated to EBV. This review article illustrates the tremendous potential of *S. cerevisiae* in integrated chemobiological approaches for the biomedical research.

|          |             |
|----------|-------------|
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**Keywords:** Cancers linked to Epstein-Barr virus (EBV) · EBNA1 · Epstein-Barr virus (EBV) · Yeast as a model for biomedical research · Yeast model for human diseases

### 1 Introduction

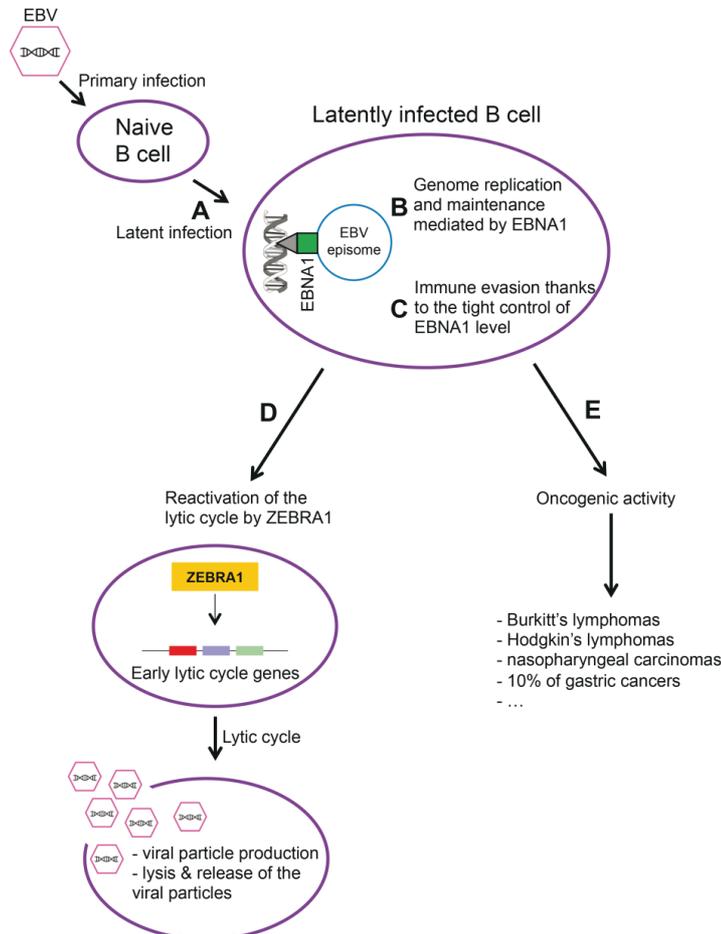
The Epstein-Barr virus (EBV) is the first oncogenic virus described in humans [1–4] and EBV is still one of the most effective means for transforming and immortalizing

human B cells. Over 90% of the population is infected by this ubiquitous gammaherpesvirus. Primary infection with EBV is normally asymptomatic but, in teenagers and adults, it can be responsible for a self-limiting lymphoproliferative disease termed infectious mononucleosis. In most individuals this virus persists as a lifelong latent asymptomatic infection but it can be responsible for severe lymphoproliferative disorders in patients suffering from some forms of immune suppression (e.g. grafted patients or HIV-infected people). EBV has also been linked to certain types of cancers ([4, 5], for a comprehensive list see [6]), the best known being the endemic form of the Burkitt's lymphoma to which the malaria infection has

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**Abbreviations:** ARS, autonomous replicating sequence; CDK, cyclin dependent kinase; CEN, centromeric sequence; CHPK, conserved herpesvirus-encoded protein kinase; DRIPs, defective ribosomal products; DXR, doxorubicin; EBV, Epstein-Barr virus; GAR, glycine alanine rich; GFP, green fluorescent protein; GMP, genome maintenance protein; HCMV, human cytomegalovirus; MHC, major histocompatibility complex; rRNA, ribosomal RNA; mRNA, messenger RNA

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**Figure 1.** Overview of the EBV life cycle. Following a primary infection of naive B-cell by EBV, a latent infection settles down in most cases (A). In this situation the virus has to replicate and maintain its genome (B) thanks to its genome maintenance protein (GMP) EBNA1 which is highly antigenic. EBNA1 is thus the Achilles heel of EBV and the virus evolved a mechanism to tightly control its level, thereby allowing the immune evasion of EBV-infected cells and, at the same time, the replication and maintenance of the viral genome (C). In a few cases, the virus is reactivated (D) which leads to the activation of the lytic cycle by ZEBRA1 and thus to the lysis of the infected B cells, thereby releasing many viral particles that can infect some more naive B-cells. Because of its oncogenic activity, EBV can also, in a few cases, promote tumors (E), in particular some lymphomas like the Burkitt's and Hodgkin's lymphomas. The budding yeast *Saccharomyces cerevisiae* has been successfully used to study all these aspects of EBV biology.

been proposed as a co-factor [3]. Other examples are the nasopharyngeal carcinoma, which is particularly frequent in Chinese and Tunisian men [7], the Hodgkin's lymphoma and 10% of the gastric cancers. The link between EBV and human diseases has been known for 50 years and as such has been the subject of extensive studies aiming at deciphering its life cycle and its role in tumorigenicity

(Fig. 1). Among these studies, the budding yeast *Saccharomyces cerevisiae* has been fruitfully used for more than 30 years, both as a model and a tool, to unravel many of the different aspects of EBV mysteries. The aim of this article is to review these uses of *S. cerevisiae* and to propose new possible applications for these yeast-based assays, as well as the creation of further yeast models dedicated to EBV.

## 2 Using yeast to explore EBV genome replication and maintenance

### 2.1 EBV genome replication

Regarding EBV, yeast has been first used from the beginning of the eighties to identify autonomous replicating sequences (ARS) in the EBV genome [8]. Indeed, the EBV genome is an extrachromosomal double-stranded circular DNA episome which uses the DNA polymerase of the host cell to replicate during latent infection in which this episomal element has to persist. Hence, this plasmidic DNA necessarily contains its own autonomous origin of replication that has been intensively searched for years without any success [9, 10]. Then, at the beginning of the eighties, the group of J. S. Pagano exploited a recently described yeast-based system [11–14] to detect ARS in the EBV episome [8]. This system (described in Fig. 2A) is based on a plasmid which contains a selective marker (usually the yeast *URA3* gene) which allows its positive selection in yeast. Importantly, this plasmid does not contain any yeast origin of replication and thus cannot confer to a *ura3*-auxotroph yeast strain the ability to grow in a medium deprived of uracil, unless an exogenous autonomous origin of replication is incorporated in the plasmid. This system has thus been used to screen eukaryotic or viral DNA fragments for their ability to self-replicate and therefore has allowed ARS identification in the EBV genome (Fig. 2A). Of note, none of the identified ARS correspond to oriP, the genetically-defined EBV latent EBNA1-dependent origin of replication which was defined shortly after [15] (EBNA1 is an EBV-encoded protein presented in the next section). They rather overlap with EBNA1-independent sites of replication identified later on by the team of C. L. Schildkraut [16, 17].

### 2.2 EBV genome maintenance

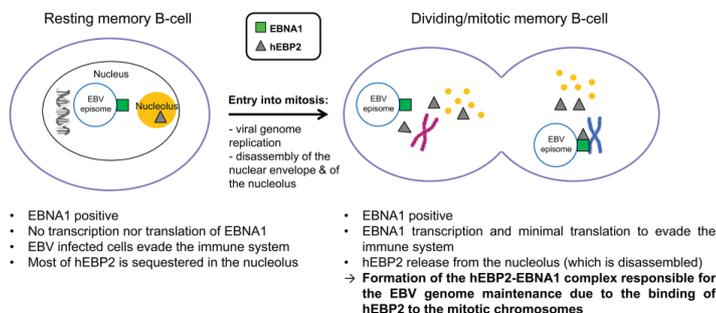
In addition to its need to replicate, the viral genome has to remain within the infected cells, which is particularly important in the case of EBV as it latently infects only a few resting B-lymphocytes which then may become highly dividing cells. The EBV genome is maintained in latently infected human cells as low copy number, double-stranded circular DNA episomes [18] which replicate once per cell cycle and are efficiently partitioned during cell division, resulting in a constant copy number per cell. Hence EBV episomes behave like minichromosomes, which implies that they contain sequences allowing their direct or indirect anchoring to the mitotic spindle or host cell chromosomes. Upon primary B-cell infection, EBV directly enters latent infection which is characterized by a reduced viral gene expression limited to a small subset of proteins termed latent antigens. These latent proteins include at least the genome maintenance protein (GMP), expression of which is crucial for the maintenance of

the viral genome in latently infected cells [19]. The GMP of EBV is the EBNA1 protein which is also essential for the efficient replication of the EBV genome. Therefore, both the replication and maintenance of EBV episomes critically depend on the virally-encoded EBNA1 protein which is expressed as such in all EBV-infected cells. In addition, EBNA1 is highly antigenic and CD8<sup>+</sup> T cells directed towards EBNA1 epitopes exist in all the infected individuals, including individuals with cancers. For these reasons, EBNA1 is widely recognized as the Achilles heel of EBV towards the immune system (see the last section dedicated to the EBNA1-based EBV immune evasion) [19]. Hence, EBNA1 is the most studied EBV-encoded protein and, at the end of the nineties, the team of L. Frappier used yeast as a tool to identify in a two-hybrid screen the human proteins that interact with EBNA1. EBNA1 was used as a bait and EBP2 (EBNA1 binding protein 2) was shown to specifically interact with EBNA1 [20]. EBP2 was also shown to bind to DNA-bound EBNA1 in a one-hybrid system and the EBP2-EBNA1 interaction was confirmed by co-immunoprecipitation. Thanks to the yeast two-hybrid system, the EBNA1 region that binds to EBP2 has been mapped to the small central glycine-arginine (GR) rich domain. The deletion of this GR domain in EBNA1 prevents EBP2 interaction and consequently the ability of EBNA1 to support the long-term maintenance of a plasmid containing the EBV origin (oriP) in human cells. Later on, a minor contribution of the small N-terminal GR of EBNA1 was also reported using a similar two-hybrid mapping [21]. More recently, the yeast two hybrid system was used to establish at the genome scale the interaction maps between EBV proteins and between EBV and human proteins [22].

### 2.3 Function of EBP2 in EBV genome maintenance

EBP2 is a 35 kDa protein conserved in eukaryotes, which led the teams of M. A. McAlear and L. Frappier to study the function of its ortholog in the budding yeast [23]. The yeast *EBP2* gene has been shown to encode an essential protein that localizes to the nucleolus and yeast cells expressing temperature-sensitive mutants of *EBP2* become depleted of ribosome and cease to divide after several generations at the restrictive temperature. A careful analysis of these mutants suggested that the EBP2 protein is involved in the processing of the ribosomal RNAs (rRNAs), thereby giving some clues about its function. Since then, the human EBP2 protein (hEBP2) has been shown to be also a nucleolar protein [24], thus probably fulfilling the same cellular function as the yeast protein. Of note is that EBNA1 was very recently shown to interact with nucleolin, another nucleolar protein essential for rRNA maturation. Nucleolin is conserved from yeast to human and its interaction with EBNA1 is critical for the genome maintenance function of the latter [25]. hEBP2 was shown by the group of L. Frappier to bind





**Figure 3.** Flowchart of the role of EBNA1/hEBP2 in EBV episome maintenance. Resting memory B-cells (left) are EBNA1 positive but no active transcription nor translation of EBNA1 (which is highly antigenic) can be detected, thereby EBV infected cells evade the immune system. At the same time most of the host cell-encoded hEBP2 protein is sequestered into the nucleolus. Dividing/mitotic memory B-cells (right) express the minimal level of EBNA1 which allows to tether the EBV episome to the host cell chromosomes. This binding also requires the host cell hEBP2 which is released from the nucleolus following its disassembly as cells enter into mitosis, thus allowing the EBNA1-hEBP2 complex to form and link the EBV episome to the host cell chromosomes. EBV has evolved a mechanism to express EBNA1 at a functional but minimal level to allow the maintenance of its episome and, at the same time, to evade the immune system.

this system, the role of the EBNA1/hEBP2 complex in the EBV genome maintenance was carefully investigated [28]. hEBP2 was found to attach to yeast mitotic chromosomes in a cell cycle-dependent manner, thus allowing the binding of the EBV episome/EBNA1 complex to the mitotic chromosome (see Fig. 3 for an integrated model). This elegant yeast-based system was thus definitively validated. Thanks to the tremendous power of yeast genetics it may further be used to decipher the cell-cycle regulated binding of EBV episomes to host cell chromosomes, a mechanism which allows their maintenance at a constant copy number in dividing cells. In this line, this yeast model has been used to highlight the importance of the phosphorylation of the four serines within the central GR rich domain of EBNA1 (the domain which interacts with hEBP2) in the regulation of the EBNA1-based segregation of EBV-derived plasmids [29]. The role of these serine phosphorylations was then validated in human cells.

### 2.5 Isolation of other host cell factors important for EBV genome segregation

Finally, the EBV-based plasmid partitioning assay also allowed the group of L. Frappier to test the ability of other host proteins, Brd2, Brd4 (which are members of the bromodomain and extra terminal domain-(BET)-family) and MeCP2 that are implicated in the genome segregation of papillomavirus and Kaposi's sarcoma-associated herpesvirus, to support EBNA1-mediated segregation of EBV-based plasmids [30]. They found that Brd4 enabled EBNA1-based segregation of the EBV genome, while Brd2 and MeCP2 had a general stimulatory effect on the plasmid maintenance. In the same study, this effect of Brd4 was subsequently validated in human cells. Of note,

in the human system the significance of this interaction appeared to be rather in transcriptional activation as opposed to EBV genome segregation. Nonetheless, it was the yeast assay that identified the Brd4-EBNA1 interaction.

### 3 Using yeast to analyze the EBV lytic cycle

As stated above, upon primary B-cell infection, EBV directly enters the latent phase. The switch from the latent phase to the lytic cycle (Fig. 1D) has been identified as a potential therapeutic target, since favoring this switch should lead to the specific lysis of EBV-carrying tumor cells (reviewed in [6]). ZEBRA is an EBV-encoded transcription factor that first drives the expression of Rta (another EBV-encoded transcription factor) and then, together with Rta, controls the expression of several early EBV lytic genes. ZEBRA is thus at the top of a cascade of events leading to the expression of EBV lytic cycle genes (Fig. 1D). ZEBRA activates the transcription by binding to ZEBRA response elements (ZREs) found in the promoters of its EBV lytic cycle target genes. ZEBRA transcriptional activity has been recapitulated in *S. cerevisiae* by the group of G. Miller which expressed ZEBRA in yeast cells that contain a reporter gene (LACZ) under the control of several ZREs [31]. This system allowed the identification of the regions of ZEBRA that play a role in the transcriptional activation of its target genes. The group of G. Miller then further showed that two natural EBV promoters are activated by ZEBRA in yeast: Zp, the promoter that regulates the expression of ZEBRA itself and EAp, the promoter controlling the expression of BMRF1, the EBV DNA polymerase processivity factor. Importantly, as yeast is a

naive system regarding EBV, these results indicate that neither mammalian-specific nor virally-encoded cofactors are mandatory for ZEBRA-based activation of the transcription from these two promoters [32].

#### 4 Using yeast to study the oncogenic activity of EBV

##### 4.1 CDK-like activity of the CHPK family of herpesviruses kinases

Altogether, the genomes of the eight known human herpesviruses, including EBV, encode at least sixteen protein kinases that are grouped in three distinct families. One of these families, the CHPK (for conserved herpesvirus-encoded protein kinase) family, comprises eight members, one from each herpesvirus. Several of these CHPKs have been shown to display a CDK-like activity by inducing the phosphorylation of the retinoblastoma (Rb) tumor suppressor protein and of the lamin A/C, two events normally catalyzed by human CDKs, thereby promoting the entry in the cell cycle and mitosis of their host cells [33, 34]. This ability most certainly accounts for the ability of the herpesviruses to promote tumor formation. UL97, the CHPK of the human cytomegalovirus (HCMV) was hence first shown to phosphorylate and thus inactivate the Rb tumor suppressor protein [35]. In this study, the budding yeast was used to demonstrate that UL97 possesses a CDK-like function. Indeed, UL97 is able to complement a defect in the function of Cdk1/Cdc28 at the G1/S transition in yeast. The essential Cdk1/Cdc28 protein is the main CDK that controls the progression through all the phases of the yeast cell cycle and various conditional (thermosensitive) mutant alleles of the *CDK1/CDC28* genes, that block yeast cells at specific stages of the cell cycle when they are grown at the restrictive temperature (36°C), have been isolated. In this study, a haploid yeast strain which contains the *cdc28-13* allele that blocks cells at the G1/S transition when grown at the restrictive temperature was used and the budding index, which reflects the ability of yeast cells to resume their cell cycle, was estimated. As expected, the expression of the human *CDK1* gene from a plasmid led to a high budding index (around 70%), as compared to the low budding index (around 40%) using an empty vector. The expression of UL97 led to a quite high budding index (around 60%) whereas a catalytically inactive form of UL97 led to the same budding index as the empty vectors (around 40%). Taken together these results show that UL97 possesses a CDK-like activity which allows yeast cells deprived of a functional Cdk1/Cdc28 to resume the cell cycle, almost as efficiently as the human CDK1, which is long known to complement yeast *cdk1/cdc28* mutants [36].

##### 4.2 Systematic analysis of the eight CHPK in yeast

In a recent study [37], the eight CHPKs have systematically been tested for their ability to complement the yeast *cdc28-13* mutant at the restrictive temperature. The EBV-encoded CDK-like proline-dependent serine/threonine kinase BGLF4 is the EBV representative of the CHPK family. BGLF4 was shown to display the highest CDK-like activity among all the CHPKs. UL97 from HCMV, and U69 from both human herpesviruses 6 and 7 (HHV-6 and HHV-7) also displayed a CDK-like activity, although to a lower extent than BGLF4, whereas other CHPKs, including UL13 from both herpes simplex viruses types I and II (HSV-1 and HSV-2), ORF47 from varicella zoster virus (VZV) and ORF36 from the Kaposi's sarcoma associated herpesvirus (KHSV) had no significant activity. Thus, according to this yeast-based assay, CDK-like activity seems to be absent in alphaherpesviruses (HSV-1, HSV-2 & VZV). Interestingly, these results fully corroborated the observations that Rb is not well phosphorylated in the cells infected by alphaherpesviruses [38], but is properly phosphorylated in the cells infected by EBV and HCMV [39, 40]. However, a more recent study did not reproduce this result [41]. In this paper, the same *cdc28-13*-based assay was used except that the yeast cells were synchronized in early S-phase using hydroxyurea prior to the shift at the restrictive temperature after which the budding index was estimated. This additional synchronization step may explain the discrepancies observed between these two studies. Furthermore, another mutated allele of the *CDC28* gene, *cdc28-4* which leads to an arrest at the G2/M transition when yeast cells are shifted at the restrictive temperature, was used to determine the ability of the various genes (human CDK1, BGLF4 and K102I, a catalytically inactive version of BGLF4) to complement the growth defect of a *cdc28-4* strain at the restrictive temperature. Only the human CDK1 was able to complement the growth defect of the *cdc28-4* strain. This test being much more stringent and based on a mutation that affects the G2/M transition, it may account for this additional discrepancy. It would clearly be interesting to fully exploit the large collection of *cdk1/cdc28* mutants to unravel the CDK-like activity of BGLF4, for example by systematically determining its ability to rescue, or not, the growth defects of these various mutant strains (see the perspective section). Finally, yeast two-hybrid approaches have allowed the identification of xeroderma pigmentosum C (XPC) [42] and a splicing variant of interferon regulatory factor 3 (IRF3) [43] as host cell interactants of BGLF4.

## 5 Using yeast to investigate the immune evasion of EBV

### 5.1 EBV strategy to evade the immune system is based on control of EBNA1 level

As stated above, most of EBV strategy to evade the immune system is based on EBNA1, its GMP. Indeed, due to its crucial role in the genome replication and maintenance, EBNA1, which is highly antigenic, has to be expressed in every EBV-infected cell. In addition, all infected individuals, including those having a cancer linked to EBV, have cytotoxic CD8<sup>+</sup> T cells directed towards EBNA1 epitopes. However, their immune system fails to detect and destroy EBV-infected cells. This is due to EBV's strategy to evade the immune system which is based on a tight control of the expression of EBNA1. EBNA1 is thus expressed at a level low enough to avoid the immune detection, but sufficient to ensure its crucial GMP function. For this reason, various teams have considered the mechanism that tightly controls the level of EBNA1, and thus responsible for its stealthiness, as a pertinent therapeutic target for EBV-associated cancers [6]. Indeed, whereas in healthy individuals the latent infection by EBV is primarily restricted to a specific pool of memory B-cells and the viral replication is spontaneously activated in only a small percentage of these cells, most tumor cells from EBV-related cancers are infected by EBV and express EBNA1 at a functional but minimal level that allows the cancer cells to evade the immune system whilst retaining the EBV episome. Therefore, overcoming the EBNA1-based mechanism of EBV stealthiness should unveil EBV-carrying tumor cells as targets for the immune system without having any significant effect on the vast majority of healthy host cells.

### 5.2 Mechanism that tightly controls EBNA1 expression

The mechanism that tightly controls EBNA1 expression has been thoroughly studied. These studies started twenty years ago when the central glycine alanine rich (GAR) domain of EBNA1 was shown to be pivotal for the ability of EBNA1 to evade the immune system [44]. EBV's GAR domain consists of single alanines separated by one, two or three glycines. The deletion of this GAR domain significantly increased the presentation of EBNA1-derived antigenic peptides by the major histocompatibility complex (MHC) class I pathway. This result was then rapidly attributed to the ability of the GAR domain to inhibit the protein degradation by the 26S proteasome [45]. Indeed, when fused to a number of known substrates of the 26S proteasome, like I $\kappa$ B $\alpha$  [45] or p53 [46], the GAR domain clearly inhibited their degradation in a length-dependent manner (the longer the GAR domain, the stronger the inhibition of the proteosomal degradation is). These

results were fully consistent with the current dogma that prevailed at that time according to which the main source of antigenic peptides comes from the proteosomal degradation of full length proteins. In line with this model, the budding yeast *S. cerevisiae* was also used to confirm that the GAR domain possesses a length-dependent ability to prevent the proteosomal degradation [47]. In this study, destabilized versions of the green fluorescent protein (GFP), harboring N-end rule or ubiquitin fusion degradation signals, were fused to GAR domains of various lengths and expressed in yeast. The length-dependent effect of the GAR domain on the protein stability was thus confirmed, suggesting either that the GAR domain possesses an intrinsic ability to inhibit the 26S proteasome, or that this effect is mediated by host cell factor(s) which is/are conserved in yeast. Of note, one curious result in this article was that, despite the GAR length-dependent stabilization of the unstable version of the GFP by the GAR domain, the steady-state level of the various GAR-GFP fusions was inversely correlated to their degree of stabilization: the longer the GAR domain, the more the reporter protein was stable but the smaller its steady state level, which appears rather counterintuitive. These discrepancies were also observed at the same time for EBNA1 as the deletion of the GAR domain from EBNA1 (EBNA1 $\Delta$ GAR) increased its level of expression instead of decreased it, as one could expect as a consequence of the deletion of a stabilization signal [48]. Finally, the model stating that the inhibition of the proteosomal degradation of EBNA1 by its GAR domain accounts for the ability of the latter to inhibit the presentation of antigenic peptides derived from EBNA1 was further challenged when it was observed that, with or without its GAR domain, EBNA1 is a very stable protein [48, 49]. Hence, even if the GAR domain is able to inhibit the proteosomal degradation of unstable proteins to which it is fused, this effect is not operant in EBNA1. In the study by Yin et al., the use of in vitro expression systems and cell-based metabolic labeling led to the demonstration that the increased EBNA1 level observed following the deletion of the GAR domain was due to an increase in the rate of EBNA1 synthesis [48]. This striking result was the starting point of other studies that demonstrated that the GAR domain is in fact an effective inhibitor in cis of the translation of any messenger RNA (mRNA) to which it is fused, without affecting the translation in trans of other mRNAs [49–53]. These results were fully in line with the DRiPs model that was at that time recently proposed by Yewdell [54, 55] and that challenged the entire concept of full length proteins being processed by the 26S proteasome to supply the MHC class I pathway with antigenic peptides. According to this model, antigenic peptides would come mainly from the DRiPs (for "defective ribosomal products"), an alternative source of peptide material that would correspond to byproducts of translation. Hence, most of the antigenic peptides would be generated during translation of mRNAs (especially

during the initiation phase). This is fully consistent with several observations among which the very rapid kinetics of the presentation of antigenic peptides by the MHC class I pathway after cell infection (a few minutes) which is not compatible with the synthesis of full length proteins followed by their degradation. In addition, inhibiting the transcription of the gene encoding the antigenic protein prevents the presentation of antigenic peptides derived from this protein during a time frame when the levels of the protein remain high [56]. Altogether, these data demonstrate that neosynthesis is required for the presentation of T cell epitopes from a long-lived viral protein.

### 5.3 A yeast model for EBV immune evasion

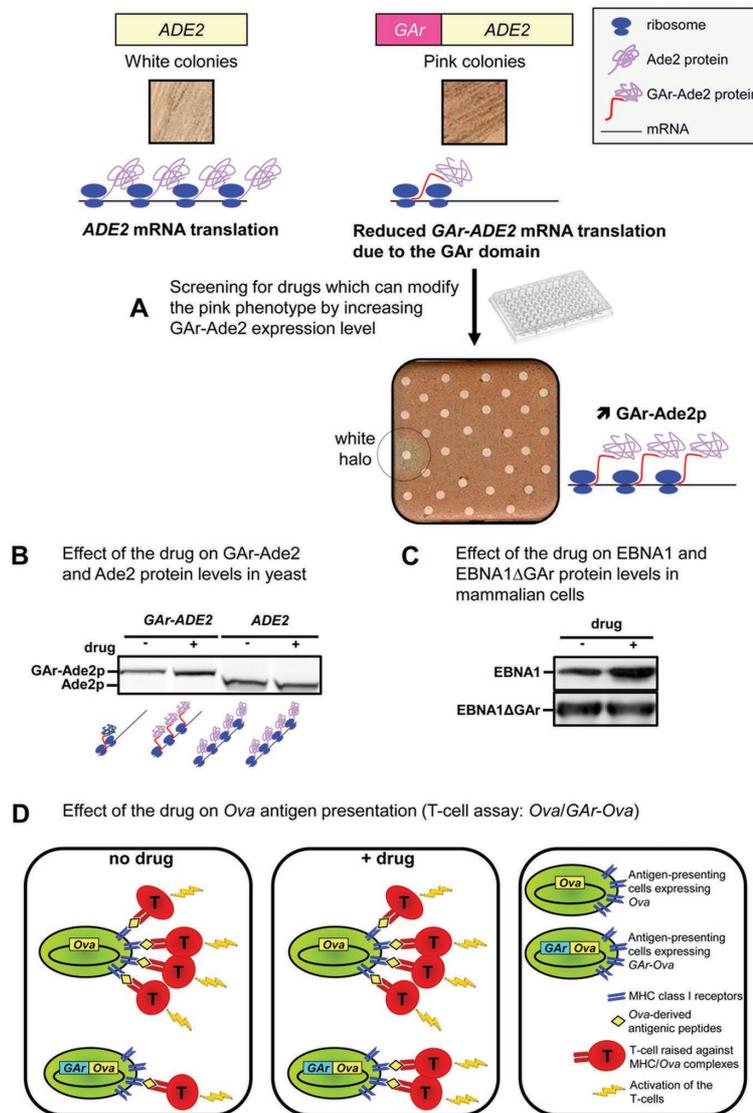
Of note, the discrepancies in the steady-state level of the various GAR-GFP fusions observed in yeast were thereby explained if, in yeast cells as in human cells, the GAR domain inhibits in cis the translation of its own mRNA in a GAR length-dependent manner. Based on this assumption, we have recently developed a yeast-based assay for monitoring the GAR domain ability to inhibit the translation of its own mRNA in cis [57]. In this model (described in Fig. 4), the yeast Ade2p protein was used as a reporter protein as: (i) it is a stable protein (like EBNA1); and (ii) a convenient colorimetric system exists to easily determine its steady state level in living cells. Indeed, whereas an *ade2Δ* strain readily forms red colonies on rich YPD medium, cells that express Ade2p at a functional level form white colonies and any intermediate level of Ade2p leads to the formation of pink colonies whose intensity of coloration is inversely proportional to the level of Ade2p expressed. Hence, any subtle change in the level of Ade2p can easily be monitored. The *ADE2* gene expressed from the constitutive *ADH* promoter, that allows its minimal expression to get white colonies, was fused to GAR domains of different lengths (21, 43 or 235 amino acids), or to the GAR domain of the Papio herpesvirus, which is known to have no effect on translation due to the insertion of one serine every seven residues of the repeat. As in human cells, the length-dependent ability of the GAR domain to inhibit the translation of its own mRNA in cis was demonstrated whereas the Papio GAR domain had no effect [57]. Thanks to the Ade2p reporter protein, the effect of GAR can easily be monitored (Fig. 4A) and served as the screening basis for identifying compounds able to interfere with its inhibitory effect on translation using a method previously described [58–60]. This way doxorubicin (DXR) was identified and shown to specifically counteract the inhibitory effect of GAR on the expression of GAR-Ade2p. This effect was GAR-dependent since DXR had no effect on the expression of Ade2p (Fig. 4B). This yeast assay was then definitively validated when DXR was not only shown to stimulate, in a GAR-dependent manner, EBNA1 expression in mammalian cells (Fig. 4C), but also to overcome the GAR-dependent restriction of MHC class

I antigen presentation (Fig. 4D) [57]. In addition, as many chemical analogs of DXR are commercially available, a structure-activity relationship study was performed using both the yeast and T-cell assays. The good correlation between the results obtained using the two assays definitively validated the yeast model for EBNA1-based EBV's stealthiness. They also indicated that the effect of DXR on the GAR-mediated inhibition of translation is uncoupled from its already known genotoxic effect [57]. What's next? The yeast assay could also be used now for various kinds of genetic screening aiming at identifying the conserved host cell factors involved in the GAR-mediated inhibition of translation and thus of antigen presentation. Of note, similarly to the drug screening, this assay should allow to identify both positive and negative regulators of the GAR effect that could then constitute attractive therapeutic targets for the treatment of cancers linked to EBV.

## 6 Concluding remarks

As with all long-lasting love affairs, there is a need to reinvent the story and thus rekindle the flame. This could be done for example by fully exploiting the yeast-based assays that already exist and model various aspect of EBV biology. Indeed, most of these assays could be used for drug screening whereas, for the time being, the yeast model for the EBNA1-based mechanism of EBV immune evasion is the only one that has been exploited this way. The possibilities were even less explored regarding genetic screenings that aim at identifying in a comprehensive manner the host cell factors necessary for the various aspects of the EBV biological cycle. In this view, exploiting the yeast models for the EBV genome maintenance and the immune evasion could certainly give some interesting clues about new mechanisms, and thus new potential therapeutic targets that are relevant for the treatment of EBV-related diseases, including cancers. In addition, regarding EBV oncogenicity, yeast strains in which the endogenous *CDK1/CDC28* gene is replaced by BGLF4, the EBV member of the CHPK family, may be constructed, first to rigorously determine if BGLF4 is a bona fide CDK-like kinase and, second, if so, to identify candidate drugs able to inhibit this activity without any significant effect on a similar yeast strain in which the endogenous *CDK1/CDC28* gene is replaced by the human CDK1. In this way specific inhibitors of BGLF4 might be identified.

Finally, one interesting aspect of the yeast models for EBV is that yeast is a "naive" eukaryote regarding EBV, considering that, contrary to human EBV host cells, no co-evolution between EBV and yeast has occurred. This obvious remark has an important consequence: even if some yeast factors could functionally substitute their human counterparts, they were for sure not evolutionary optimized, which means that their overexpression should



**Figure 4.** Principle of the yeast-based assay for EBNA1-based immune evasion of EBV. (A) The GAR domain of EBNA1 which is responsible for its self-limiting expression (thereby allowing the immune evasion of EBV) was fused to the yeast *ADE2* reporter gene which encodes Ade2p, a very stable protein, like the EBNA1 protein. Full expression of Ade2p in yeast leads to the formation of white colonies, whereas, due to the inhibition of its translation in cis by the GAR domain, the *GAR-ADE2* construct leads to the formation of pink colonies. This effect, which is GAR length-dependent as in human cells, demonstrates that the ability of the GAR domain to inhibit the translation of its own mRNA is also operant in yeast. This assay based on the pink phenotype of the GAR-Ade2p expressing yeast cells was used to perform drug screening aiming at isolating compounds able to interfere with the GAR effect on translation (A). Drugs that suppress the inhibitory effect of GAR on translation lead to the formation of whiter colonies that are easily monitored on Petri plates. The effect of these drugs on the GAR-dependent inhibition of the protein expression can then be evaluated both in yeast (B) and in human cells (C). Finally, their effect on the GAR-mediated restriction of the antigen presentation can also be evaluated using a T-cell assay (D). At each step it is important to check if the effects observed are GAR-dependent.

in principle enhance the efficiency of the considered EBV-related process, which makes them easier to detect in global approaches than using approaches based on their inactivation which are more tedious, even in yeast [61].

In conclusion, many new exciting yeast-based adventures to decipher EBV biology are still to come, thus promising some rejuvenation of the long-lasting love story between the budding yeast and the Epstein-Barr virus. In addition the success of this love story may inspire similar yeast-based stories to study other gammaherpesviruses also involved in human diseases, in particular some cancers, like the Kaposi and papilloma viruses. Hence, despite the fact that it has been used for decades and that one may have initially considered it as an unlikely match, the yeast-EBV couple is still as glamorous as at its beginnings, and will certainly keep playing a key role in deciphering some of the EBV mysteries still to be discovered.

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*The authors declare no financial or commercial conflict of interest.*

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**Marc Blondel, María José Lista and Cécile Voisset** work in the YMCD Inserm UMR1078 team at the University School of Medicine in Brest, France. Their main focus of interest

is to develop yeast (*Saccharomyces cerevisiae*)-based chemobiological models for various human diseases that include prion-based diseases, mental retardation observed in the Down syndrome or cancer linked to infection by the Epstein-Barr virus. Once established, these models are used to isolate modifiers that can either be genes to decipher cellular mechanisms involved in the disease of interest, or drugs that can be developed toward a use in the clinic. Hence, yeast is a starting point which is used both as a tool and a model in these chemobiological approaches.



**Frédéric Bihel** works in the department of medicinal chemistry (CNRS, University UMR7200) at the Faculty of Pharmacy in Strasbourg. His main focus of interest is to develop new chemical entities based on heterocyclic scaffolds targeting various human diseases that include neurodegenerative disorders or cancers. He also develops pharmacological tools (biotin, fluorescent

probes, etc) designed to get an insight in the biological mechanisms involved in these pathologies.



The work of the team of **Robin Fähræus and Chrysoula Daskalogianni** in Inserm UMR1162 at Hôpital Saint Louis in Paris has been focused on different aspects of basic cancer research and more recently on the physiological aspects of mRNA translation control

in the p53 and in the MHC class I restricted pathways. They are interested to see how signaling pathways use mRNAs within the p53 pathway to help differentiate p53 tumor suppressor activity. They are also interested to know the physiological importance during cancer and viral infection of an alternative source of peptides for the MHC class I pathway that is produced during the processing and maturation of the nascent mRNA.

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#### Cover illustration

This regular issue of BTJ features articles on the production of biofuels, small molecules and recombinant proteins. The cover is inspired by an article describing increased expression levels of recombinant proteins in potato tubers upon post-harvest light treatment. © Lenslife – Fotolia.com

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<http://dx.doi.org/10.1002/biot.201500581>

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Research Article

**Post-harvest light treatment increases expression levels of recombinant proteins in transformed plastids of potato tubers**

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Biotech Method

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*Jens Begemann and Antje C. Spiess*

<http://dx.doi.org/10.1002/biot.201500198>

# Discussion

Oncogenic viruses have evolved several strategies which allow them to escape from host immune system recognition and elimination. They have developed various mechanisms that target all the steps of the MHC class I and class II pathways. They can affect MHC class I synthesis and degradation, peptide-loading, peptide transport into the ER, mRNA translation, proteasome degradation, etc. As presented in the introduction, EBV expresses different proteins during the latent phase and the lytic cycle in order to avoid MHC class I and class II responses. During latency, the EBNA1 protein is able to inhibit its own mRNA translation *in cis*, a process led by its central GAR domain, and in that way reduces the production of EBNA1-derived antigenic peptides and the further recognition of infected cells by EBNA1 specific T cells<sup>91</sup>. When I started this PhD, the mechanism involved in this process was undeciphered, and two hypotheses that are not mutually exclusive were ventured:

- The team of R. Fåhræus showed evidence supporting a model by which the GAR nascent polypeptide would block the translation of the upcoming mRNA. They demonstrated *in vitro* that the polysomal profile of the EBNA1 mRNA mimics the one of EBNA1ΔGAR in which they have introduced a hairpin secondary mRNA structure in the 5'-UTR<sup>5</sup>, suggesting that GAR may block the translation initiation process. In line with this observation, the insertion of the c-MYC IRES into GAR-OVA mRNA completely abolishes the GAR inhibition of translation effect, suggesting that this mechanism depends of the CAP-dependent translation initiation.
- The team of J. Tellam showed that the GAR mRNA forms G4 structures that seem to be responsible for the ribosomes stalling and thus block the translation elongation process<sup>99</sup>. Furthermore, frameshifts of EBNA1 internal repeat altering protein sequence but not mRNA sequence were still able to inhibit their own mRNA translation and antigen presentation<sup>93</sup>. In addition, the treatment with small G4 ligands (for example PDS) that stabilize G4s was shown to decrease EBNA1 translation in an *in vitro* transcription-translation coupled system. Hence, this hypothesis is only based on *in vitro* experiments.

When I started my PhD, these two hypotheses had in common the fact that the cellular factors involved in this GAR-dependent regulation of translation were unknown.

In order to identify such cellular factors no matter the mechanism involved, we performed a genetic screening using the yeast-based assay that recapitulates GAR translation inhibition (**Figure 13**). This

approach allowed us to identify the first cellular factor that is involved in GAR-based translation inhibition and immune evasion: Nucleolin (NCL).

*1. Identification of NCL, the first cellular factor able to affect the GAR-based EBNA1 translation inhibition*

We proved that NCL overexpression in EBV-infected cells enhances EBNA1-dependent regulation of translation and inversely that its downregulation by siRNA increases EBNA1 translation (**Figure 2, chapter 1 of the results section**). An increase in EBNA1 translation may constitute a therapeutic solution against EBV-associated cancers, since it also increased the antigenic peptides presented at the surface of APC and also specific T CD8<sup>+</sup> proliferation (**Figure 3, chapter 1 of the results section**). This means that virus-infected cells would be unveiled to T cells directed towards EBNA1 which exist in all EBV-infected individuals.

The relationship between NCL and EBV was showed for first time by the group of YL. Chen that proved that the RNA binding domains (RBDs) of NCL are able to interact with the N-terminal domain of the EBNA1 protein and that this interaction enhances EBNA1-mediated OriP transcription. These studies where made in EBV negative cells which were transiently transfected with flag-tagged EBNA1. Co-immunoprecipitation assays showed the association between NCL and EBNA1, and the authors concluded that NCL/EBNA1 association is not dependent on the presence of DNA or RNA. The authors showed that NCL overexpression in these cells significantly increased the ability of EBNA1 to activate OriP transcription. They also claimed that NCL overexpression does not affect EBNA1 protein levels<sup>145</sup>. However, the published results do not fit with their conclusions as their data clearly show that NCL overexpression decreases EBNA1 levels by 50%. Two years later the same authors demonstrated that another cellular factor, the ribosomal protein L4 (RPL4), is also implicated in EBNA1 OriP transcription activation<sup>146</sup>. Altogether our data and Chen's group data show that NCL is highly linked to at least two of EBNA1 functions: transcription enhancement and immune evasion. The identification of NCL as a GAR mRNA binding protein represents another argument in favor of the G4 hypothesis, but does not exclude a possible involvement of the GAR polypeptide.

We demonstrated that NCL-GAR RNA interaction takes place in the nucleus (**Figure 4, chapter 1 of the results section**). Nevertheless, we still don't know how this interaction occurs and what the exact mechanism is. Three different scenarios are possible:

- first, the interaction between NCL and GAR mRNA may trap the GAR mRNA in the nucleus, thereby preventing its translation by the cytoplasmic ribosomes.
- second, GAR G4 motifs may act as a recognition signal for NCL which interaction with G4 is able to directly affect translation initiation and/or elongation.
- third, NCL may act as a stabilizer of the G4s motif which are themselves able to inhibit translation.

These hypotheses are equally possible and not mutually exclusive. No matter the mechanism, it is unlikely that the virus has developed a new bright mechanism involving NCL in order to control its own EBNA1 RNA translation. More probably, the virus is using a mechanism that already exists in the cells. Furthermore, the fact that GAR-based translation inhibition works in yeast reinforces the hypothesis of an evolutionary conserved mechanism.

## *2. How does NCL impairs the GAR-based EBNA1 translation inhibition?*

To get deeper into GAR RNA-NCL interaction, and since this interaction takes place in the nucleus, we wondered what may happen if we mislocalize GAR RNA from the nucleus to the cytoplasm. Using proximity ligation assay (PLA) and RNA co-immunoprecipitation we demonstrated that the mislocalization of GAR RNA to the cytoplasm abolishes its interaction with NCL (**Figure 2, chapter 2 of the results section**).

Furthermore, the cytoplasmic localization of GAR RNA totally abolishes the GAR effect on the regulation of translation (**Figure 3, chapter 2 of the results section**). In line with this observation, we remarked that RNAs that are naturally targeted to the cytoplasm are not subject to GAR translation inhibition. However, the retention of these pre-RNA in the nucleus allows their interaction with NCL in the nucleus (**Figure 4H, chapter 2 of the results section**), as well as the decrease of the translation of these RNAs and the reduction of antigenic presentation.

These results indicate that NCL interaction is essential in order to ensure GAR-based translation inhibition, whereas it seems that the nuclear localization of GAR RNA is due to the fact that NCL is mostly present in the nucleolus and the nucleoplasm. In order to reinforce our hypothesis, we constructed a NCL mutant which nuclear localization signal (NLS) is mutated (NCL mutNLS), and that is consequently unable to return to the nucleus once synthesized (**Figure 5A, chapter 2 of the results section**). We demonstrated that the cytoplasmic NCL mutNLS is able to interact with GAR RNAs that

have been relocalized in the cytoplasm (**Figure 5C, chapter 2 of the results section**), and that this interaction affects the GAR-based translation inhibition (**Figure 5D-E, chapter 2 of the results section**). We showed that NCL binding to GAR RNA is sufficient to ensure GAR-based inhibition of translation. Taken together the results obtained in the second part of this thesis showed that NCL interaction with GAR RNA is essential to ensure GAR-based translation inhibition.

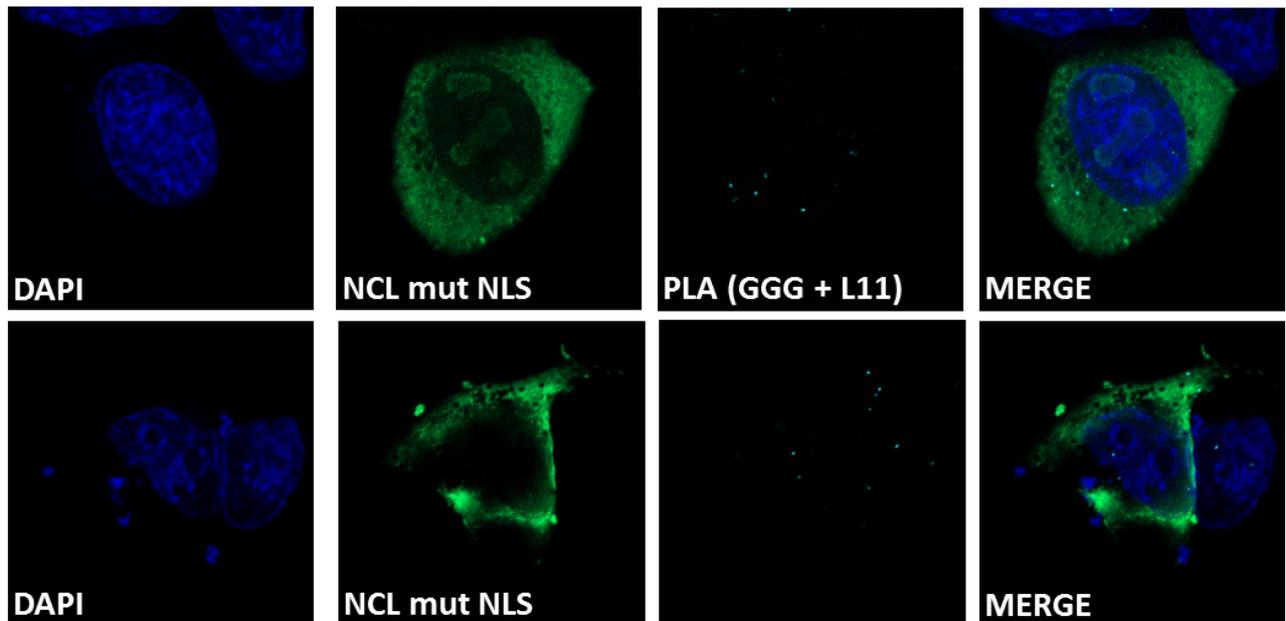
The results presented in this second chapter showed for the first time that EBV escapes from the immune system recognition during the latent phase by taking advantage of a common cellular mRNA motif (G4) that recruits NCL thereby controlling the levels of EBNA1 expression.

Nevertheless, we have not determined yet if GAR RNA retention in the nucleus by NCL is sufficient enough to inhibit translation or if its own inhibitory effect through G4 binding is also necessary.

Moreover, we still don't know how NCL inhibits translation: does NCL binding to GAR RNA block the binding of the ribosomal subunit 40S, the ribosomal assembly of the 60S or the ribosome progression? Another possibility is that the GAR polypeptide could also be involved in this interaction and thus the complete mechanism of translation inhibition would implicate the triple interaction between GAR mRNA, GAR polypeptide and NCL.

To decipher if ribosome assembly takes place when NCL interacts with GAR RNA, we determined if cytoplasmic GAR RNAs are able to interact with the ribosomal protein L11 when a cytoplasmic version of NCL is expressed. Our preliminary results show that cytoplasmic GAR RNAs are able to interact with the ribosomal protein L11 that is associated to the large subunit of the ribosome. These data suggest that the ribosome is probably assembled on GAR mRNA but that translation initiation and/or elongation is blocked by NCL binding (**Figure 14**). Some more experiments will help confirming these results.

Another intriguing question that raised on concerns the timing of the interaction between NCL and GAR G4s. A regulation is most probably controlling this mechanism in order to permanently ensure a minimal level of EBNA1 which is essential for the episome replication and maintenance.



**Figure 14-** Nucleolin binding to GAR-carrying RNAs doesn't affect ribosomal assembly.

Proximity ligation assay carried out between GGG RNA and L11 combined with NCL $\Delta$ NLS immunostaining showed that GAR-carrying RNAs present in the cytoplasm are able to interact with the ribosomal protein L11, and that this interaction co-localized with the location of cytoplasmic NCL $\Delta$ NLS.

In normal conditions most of the available NCL is localized in the nucleolus where it accomplishes crucial functions among which ribosome biogenesis. During cell division, the nucleolus is disrupted and re-generated after mitosis, which frees all NCL that was trapped in the nucleolus. At this stage of cell division, EBNA1 needs to be produced to ensure the transmission of the viral genome to daughter cells by tethering the viral episome to the mitotic host chromosomes. However, the uncontrolled EBNA1 high rate of expression would lead to the increase of EBNA1-derived antigenic peptides and thus the recognition of infected cells by specific T CD8<sup>+</sup>. For this reason, NCL-GAR RNA interaction should likely take place when EBNA1 translation rate is higher in order to avoid the recognition and elimination of EBV-infected cells. It would be interesting to challenge this hypothesis by performing a PLA assay between NCL and GAR RNA at different steps of the cell cycle of synchronized cells.

Since NCL is subjected to several post-translational modifications which may control its localization in the cell, another possibility is that a particular population of NCL (eg, phosphorylated NCL) which is localized in the nucleoplasm may be interacting with GAR G4 motifs. It would be interesting to try to identify this population of NCL by performing a G4 pull-down experiment followed by the detection of NCL with antibodies that can reveal NCL phosphorylation's or acetylation's.

To conclude, we described a novel function of NCL in EBV latency during which the interaction of NCL with EBNA1 GAr mRNA is directly implicated in translation inhibition and hence responsible for the poor recognition of infected cells by T cells directed against EBNA1. These data suggest that targeting GAr mRNA-NCL interaction constitutes a novel therapeutic target to treat EBV-associated cancers.

In the light of our data showing the nuclear localization of EBNA1 mRNA and the cytoplasmic export driven by c-MYC IRES, another possible interpretation would be that c-MYC IRES-carrying mRNAs have no GAr-based translation inhibition because they are exported to the cytoplasm (see introduction section 2.3.1).

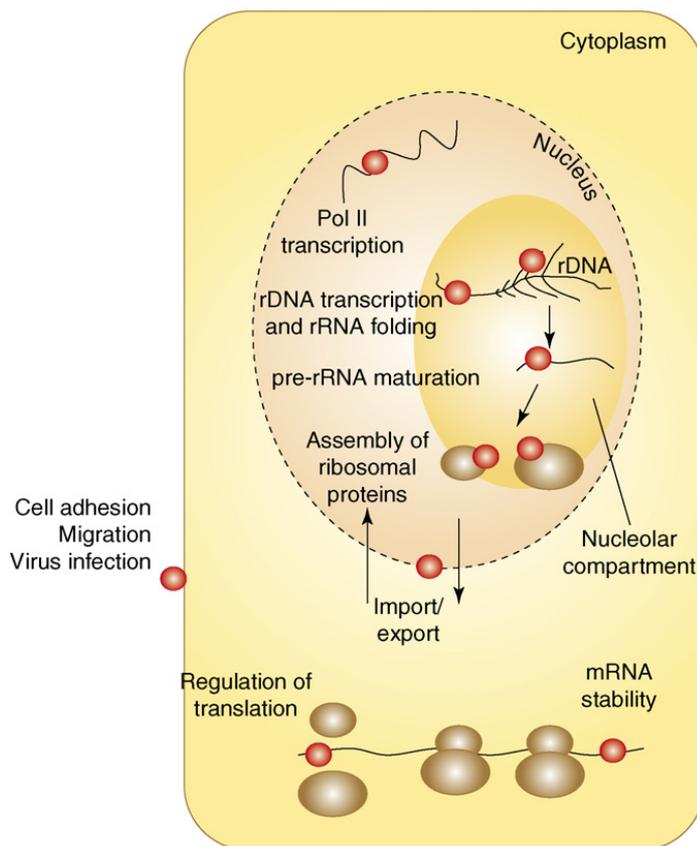
### 3. *Targeting GAr mRNA-NCL interaction*

In order to disrupt GAr mRNA-NCL interaction, both interactors can be targeted. Since NCL is essential and implicated in several cellular pathways, targeting NCL is probably not the best option. We observed that NCL overexpression is often toxic for cells, and inversely its knockdown is only about 40-50%, meaning that NCL cell levels should stay mostly stable for cell viability. On the other hand, targeting G4s motifs should be easier, but since these motifs are present all over the human genome there is a selectivity issue, as modifying G4 stability could also affect several steps in gene regulation.

#### - **Targeting NCL:**

Due to its numerous cellular functions, NCL has been associated to cancer development and progression and also to neurodegenerative disorders. During tumorigenesis NCL is involved in several steps: first, its overexpression causes an increase in rRNA synthesis and ribosome biogenesis in the nucleolus. Second, NCL is able to enhance oncogene expression which leads to anti-apoptotic and cell survival signals.

However, no NCL mutations or splicing variants have been identified yet. Nevertheless, NCL localization (**Figure 15**) is often modified and associated with several human pathologies as cancer. It has been shown that in many types of human cancers (like gastric cancers, melanomas, glioblastomas), NCL is overexpressed and mislocalized to the nucleoplasm, cytoplasm or the cell surface <sup>147</sup>. For example, in gastric cancer, NCL is highly overexpressed and the accumulation of cytoplasmic NCL is associated with a poor prognosis for patients. When situated at the cell surface,



NCL is able to interact with several cellular proteins that help with the tumorigenesis process. In B lymphocytes, NCL interacts with Fas and blocks the interaction with FasL, causing a decrease in apoptosis. Furthermore, in glioblastoma a glycosylated form of cell surface NCL is associated with the grade of cancer malignancy, indicating that different post-translational modifications of NCL can be involved in different aspects of the oncogenic process <sup>148</sup>.

**Figure 15-** The different cellular locations of NCL. Figure extracted from <sup>1</sup>.

The red dots represents NCL at its different locations. In the nucleolus NCL association with chromatin (rDNA) could regulate Pol I transcription. Also, NCL binding to pre-rRNA affects its maturation and its assembly with ribosomal proteins. Furthermore, the shuttling of NCL between the nucleus and the cytoplasm might participate in the transport of several ribosomal proteins. In the nucleoplasm, NCL has been associated to various mRNAs affecting their translation or stability. Finally, NCL has also been identified at the cell surface where it can affect cellular migration and adhesion, as well as viral infection.

Because of this, targeting NCL has been considered as a novel therapeutic target against cancer over the past few years. Nevertheless, NCL targeting is not an easy task. The G-rich aptamer AS1411 which is currently in phases I and II of clinical trials, is promising for therapy as it is able to target different

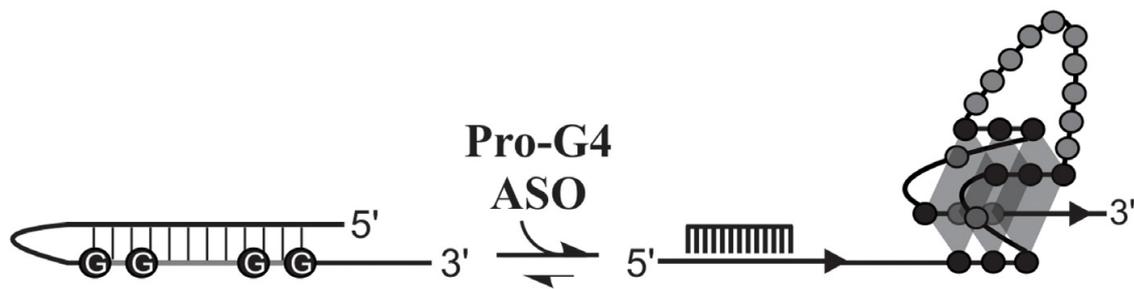
NCL signaling pathways. The overexpression of the miRNAs miR-21, miR-103, miR-221 and miR-222 is often associated with aggressiveness in breast cancer. It has been shown that NCL is able to interact with the microprocessor complex and enhance the production and maturation of these miRs. The treatment with AS1411 inhibits the interaction of NCL with the microprocessor complex which indeed reduces the production of these miRs thus leading to the upregulation of genes involved in apoptosis<sup>149</sup>. AS1411 also blocks the interaction of cytoplasmic NCL with many other mRNAs like the anti-apoptotic Bcl2 mRNA thus inducing apoptosis in cancer cells. Most interesting, AS1411 is able to specifically bind and trap cell surface NCL avoiding its interaction with key proteins. This is the case in HIV-1 infection where AS1411 treatment dramatically decreases HIV-1 attachment to the cell, which makes this G-rich aptamer a potential drug against HIV-1 infection<sup>133</sup>.

We performed a preliminary experiment treating 235GAr-OVA and OVA expressing cells with 10  $\mu$ M of AS1411 in order to determine if it has any effect on 235GAr-OVA expression (data not shown). As expected, since GAr mRNA-NCL interaction occurs in the nucleus, AS1411 has no effect on trapping the NCL population present in the nucleus and thus AS1411 treatment has no effect on GAr-based translation inhibition.

- **Targeting G4 structures:**

In order to target G4 structures, two different ways have been ventured: first, the use of antisense oligonucleotides that may affect the stability of the structure; second, the use of small G4 ligands that would stabilize or destabilize the motif. Antisense oligonucleotides (ASO) targeting G4s have been successfully used by the group of J.P Perreault. For example, the histone variant H2AFY (which is often overexpressed in cancer) contains a G-rich tract in its 5'-UTR which should be able to form G4. However, the G4 motif cannot be formed because the G-rich tracts are already engaged in a Watson-Crick-based stable structure (**Figure 16**).

The group of J.P. Perreault showed using cell-based assays that an ASO that is complementary to the G-tracts upstream region can promote the release of the G-tracts and thus allow their folding into a G4 motif<sup>150</sup> which will then inhibit the translation of H2AFY.



**Figure 16-** Promoting G4 folding of the histone variant H2AFY. Figure extracted from <sup>150</sup>.

Graphical representation of the ASO-based strategy used to promote the folding of the G4 motif present inside the 5'-UTR of the histone variant H2AFY. Under normal conditions the structure cannot be formed because the G-tracts are already part of another nucleotide structure.

Furthermore, the group of P. Murat also used an ASO to target the GAR domain G4s. They showed that it abolishes G4 formation and thus causes an increase in EBNA1 translation *in vitro* <sup>99</sup>. It will be interesting to test the ability of this ASO to disrupt NCL binding to EBNA1 RNA.

On the other hand, we also tested several small G4 ligands predicted to stabilize these structures and which should decrease EBNA1 translation. First, we tested pyridostatin (PDS) which has already been tested by the group of J. Tellam *in vitro*. They showed that PDS treatment specifically decreases EBNA1 translation by stabilizing GAR G4s motif <sup>99</sup>. Unfortunately, *in cellulo* we didn't see any effect of PDS treatment in cell-based systems (**Figure 5A, chapter 1 of the results section**). This can be due to the fact that P. Murat's group only performed these experiments *in vitro* which conditions are very different in comparison to cell-based assays. Next we tested several other compounds among which PhenDC3. Surprisingly, PhenDC3 treatment enhances 235GAR-OVA and endogenous EBNA1 expression contrary to what was expected (**Figure 5B, D-E, chapter 1 of the results section**). We showed that PhenDC3 competes with NCL for binding to GAR domain G4s (**Figure 5C, chapter 1 of the results section**). Furthermore, PhenDC3 treatment also increases T CD8<sup>+</sup> proliferation (**Figure 6, chapter 1 of the results section**). These results constitute the proof of concept that NCL-GAR mRNA interaction is druggable and thus represents novel therapeutic target to treat EBV-associated cancers. In addition, we tested other G4 ligands (data not shown) and observed that some of them have a stabilizing effect (thus enhancing GAR-dependent translation inhibition effect), whereas some others display a similar destabilization effect as PhenDC3, and some others have no effect at all on GAR translation inhibition, showing that these ligands can have different effects. However, G4 motifs are present all over the human genome, meaning that the treatment with G4 ligands can potentially

affect other cellular pathways and also the binding of NCL to key DNA or RNA G4s, as for example to the DNA G4s present in the oncogene c-Myc which can have an effect on cancerogenesis. Hence, it could be interesting to develop transcriptomic analysis in order to determine the effect of PhenDC3 on the transcription of cellular genes and also of EBV genes. In addition, it could be interesting to design a new G4 molecule able to specifically affect GAr mRNA G4-NCL interaction without affecting NCL binding to other targets.

In conclusion, in order to prevent GAr mRNA-NCL interaction, the better option seems to target the formation of the G4s present inside the GAr-encoding mRNA, either by the use of ASO or small G4 ligands. Of note, the use of ASO seems to be more specific for the different G4s motif and thus would avoid the recognition of other G4s present inside the human genome.

#### 4. *NCL implication in other viral infections*

Several human viruses like HIV-1 or HPV are also able to specifically interact with NCL, this interaction being involved in viral entry and infection. This is the case in cervical cancer where NCL overexpression enhances the expression of the oncogenic proteins E6 and E7 from HPV<sup>151</sup> which are involved in tumor growth and malignancy, and inhibition of the tumor suppressor genes pRb and p53. In addition, NCL interacts and stabilizes the G4 motifs present in the LTR promotor of HIV-1 and inhibits the transcription of the integrated provirus<sup>131</sup>. Furthermore, cell surface NCL acts as a receptor of HIV-1 and favors cell attachment and entry<sup>134</sup>. Moreover, HIV-1 infected patients present a hyperlocalization of NCL at the surface of infected cells<sup>134</sup>.

These results suggest a common mechanism between human viruses which take advantage of a ubiquitous and essential cellular protein and the formation of a particular mRNA structure in order to favor viral entry, replication, transcription or translation of viral proteins. In the case of EBV, the GAr domain G4s would act as a recognition signal for NCL binding in order to ensure a minimal synthesis level of EBNA1 protein to assure EBV episome replication and maintenance, but low enough to avoid recognition of EBV-infected cells by specific T cells.

5. *Is NCL interaction with GMPs mRNAs a common mechanism of oncogenic herpesviruses to avoid immune system elimination?*

As described above, NCL was reported to be capable of interfering with viral infections. Due to its numerous functions in various cellular pathways, viruses have taken advantage of NCL binding in order to favor virus cell cycle and infection. In particular, the two human  $\gamma$ -herpesviruses EBV and KSHV have exploited NCL binding in different ways. As stated above, the N-terminal domain of the EBNA1 protein of EBV interacts with NCL which allows the OriP-mediated transcription of the viral episome. In the case of KSHV, this virus expresses the endonuclease SOX that promotes the degradation of cytoplasmic mRNAs during lytic infection. Some mRNAs need to be protected in order to ensure viral persistence. One of these protected mRNAs is the interleukine-6 (IL-6), a growth factor essential for viral maintenance in infected B cells. M. Muller *et al* demonstrated that after KSHV infection, NCL was re-localized from the nucleolus to the cytoplasm where it can interact with IL-6 mRNA 3'-UTR and mediate its protection against SOX<sup>152</sup>. Despite these findings nothing has been previously demonstrated regarding NCL interaction with viral proteins during the latent phase. Since the GMPs of KSHV (LANA1), and EBV (EBNA1) present high homology in terms of nucleic acids, and the fact that predicted G4 have been identified in the GAR-like domain of LANA1 (QED domain, **Figure 17B**), one could assume that the mechanism involved in LANA1 translation inhibition could also involve NCL binding. In line with this hypothesis, it could be interesting to test the effect of NCL downregulation on the level of LANA1 expression and antigenic presentation, which would constitute also a novel therapeutic target to treat cancers associated to KSHV which are particularly common in some parts of the world.

Moreover, Baboon and Rhesus EBNA1-like GMPs (EBNA1ba and EBNA1rh respectively) are able to accomplish EBNA1 function in genome replication and maintenance but their GAR domains have no effect on translation inhibition. This could be explained since both EBNA1rh and EBNA1ba contain shorter GAR-like domains (**Figure 17C-D**), and although their repetitive sequences are able to form putative G4 sequences, it has not been proven yet if they contain G4 and if they are able to interact with NCL. It could be interesting to test NCL ability to interact with these GAR-like mRNA sequences in order to understand why these domains are not capable of inhibiting mRNA translation and antigen presentation.

**A**

| Position | Length | QGRS                                  | G-Score |
|----------|--------|---------------------------------------|---------|
| 13       | 17     | <u>GGAGCGGGAGGGGCAAG</u>              | 21      |
| 34       | 11     | <u>GGAGGGGCAAG</u>                    | 19      |
| 49       | 20     | <u>GGAGGAGGGGCAAGAGCAGG</u>           | 21      |
| 73       | 30     | <u>GGGGCAGGAGGGGCAGGAGGGGCAGGAGGG</u> | 42      |
| 112      | 20     | <u>GGAGGAGGGGCAAGAGCAGG</u>           | 21      |
| 133      | 29     | <u>GGAGGGGCAAGAGGGGCAAGAGGGGCAAG</u>  | 21      |
| 166      | 20     | <u>GGAGGAGGGGCAAGAGCAGG</u>           | 21      |
| 196      | 29     | <u>GGAGGGGCAAGAGCAGGAGAGGGGCAAG</u>   | 21      |
| 228      | 21     | <u>GGCAGGAGGGGCAAGAGCAGG</u>          | 20      |
| 253      | 20     | <u>GGGGCAGGAGCAGGAGGAGG</u>           | 21      |
| 273      | 21     | <u>GGCAGGAGGGGCAAGAGCAGG</u>          | 20      |
| 295      | 29     | <u>GGAGGGGCAAGAGGGGCAAGAGGGGCAAG</u>  | 21      |
| 328      | 10     | <u>GGAGGAGGGG</u>                     | 20      |
| 346      | 29     | <u>GGAGGGGCAAGAGGGGCAAGAGGGGCAAG</u>  | 21      |
| 379      | 17     | <u>GGAGGGGCAAGAGCAGG</u>              | 19      |
| 397      | 29     | <u>GGAGGGGCAAGAGGGGCAAGAGGGGCAAG</u>  | 21      |
| 434      | 18     | <u>GGGCAAGGAGCAGGAGGG</u>             | 20      |
| 463      | 19     | <u>GGGGCAGGAGCAGGAGGGG</u>            | 20      |
| 484      | 29     | <u>GGAGGGGCAAGAGCAGGAGGGGCAGGAGG</u>  | 21      |
| 513      | 17     | <u>GGCAGGAGCAGGAGGGG</u>              | 19      |
| 532      | 29     | <u>GGAGGGGCAAGAGCAGGAGAGGGGCAAG</u>   | 21      |
| 568      | 29     | <u>GGAGCAGGAGAGGGGCAAGAGGGGCAAG</u>   | 21      |
| 610      | 29     | <u>GGAGGGGCAAGAGCAGGAGGGGCAGGAGG</u>  | 21      |
| 649      | 29     | <u>GGAGGGGCAAGAGGGGCAAGAGCAGGAGG</u>  | 21      |
| 679      | 19     | <u>GGGGCAGGAGCAGGAGGGG</u>            | 20      |

**B**

| Position | Length | QGRS                                 | G-Score |
|----------|--------|--------------------------------------|---------|
| 24       | 20     | <u>GGATGATGAGGAGGAGCAGG</u>          | 15      |
| 54       | 14     | <u>GGAGGACGAGGAGG</u>                | 18      |
| 75       | 17     | <u>GGAGGATGACGAGGAGG</u>             | 15      |
| 99       | 17     | <u>GGAGGATGACGAGGAGG</u>             | 15      |
| 135      | 17     | <u>GGAGGATGACGAGGAGG</u>             | 15      |
| 159      | 14     | <u>GGAGGAGGACGAGG</u>                | 18      |
| 174      | 29     | <u>GGAGGACGAGGAGGAGGAGGACGAGGAGG</u> | 21      |
| 204      | 11     | <u>GGAGGAGGAGG</u>                   | 21      |
| 237      | 20     | <u>GGACAATGAGGACGAGGAGG</u>          | 15      |
| 258      | 29     | <u>GGACAAGAGGAGGACGAGGAGGACGGGG</u>  | 21      |
| 654      | 29     | <u>GGAGGAGCAGGAGCAGCAGGAGGAGCAGG</u> | 21      |
| 690      | 17     | <u>GGAGGAGCAGGAGCAGG</u>             | 18      |
| 714      | 23     | <u>GGAGCAGGAGCAGGAGTTAGAGG</u>       | 18      |
| 741      | 23     | <u>GGAGCAGGAGTTAGAGGAGCAGG</u>       | 18      |
| 777      | 23     | <u>GGAGCAGGAGCAGGAGTTAGAGG</u>       | 18      |
| 825      | 23     | <u>GGAGCAGGAGTTAGAGGAGCAGG</u>       | 18      |
| 852      | 23     | <u>GGAGTTAGAGGAGCAGGAGCAGG</u>       | 18      |
| 882      | 23     | <u>GGAGCAGGAGCAGGAGTTAGAGG</u>       | 18      |
| 909      | 23     | <u>GGAGCAGGAGTTAGAGGAGCAGG</u>       | 18      |
| 936      | 23     | <u>GGAGTTAGAGGAGCAGGAGCAGG</u>       | 18      |
| 966      | 23     | <u>GGAGCAGGAGCAGGAGTTAGAGG</u>       | 18      |
| 993      | 23     | <u>GGAGCAGGAGTTAGAGGAGCAGG</u>       | 18      |
| 1029     | 23     | <u>GGAGCAGGAGCAGGAGTTAGAGG</u>       | 18      |
| 1056     | 23     | <u>GGAGCAGGAGTTAGAGGAGCAGG</u>       | 18      |
| 1083     | 23     | <u>GGAGTTAGAGGAGCAGGAGCAGG</u>       | 18      |
| 1113     | 20     | <u>GGAGCAGGAGCAGGAGCAGG</u>          | 21      |
| 1140     | 29     | <u>GGAGGTGGAGAGCAAGAGCAGGAGCAGG</u>  | 12      |
| 1185     | 11     | <u>GGAGGTGGAGG</u>                   | 21      |
| 1212     | 29     | <u>GGAGGAGCAGGAGGAGCAGGAGTTAGAGG</u> | 21      |
| 1245     | 29     | <u>GGAGGAGCAGGAGGAGCAGGAGTTAGAGG</u> | 21      |
| 1278     | 29     | <u>GGAGGAGCAGGAGGAGCAGGAGTTAGAGG</u> | 21      |
| 1311     | 26     | <u>GGAGGAGCAGGAGCAGGAGGGGTGG</u>     | 18      |
| 1344     | 17     | <u>GGAGCAGGAGCAGGAGG</u>             | 18      |

**C**

| Position | Length | QGRS                         | G-Score |
|----------|--------|------------------------------|---------|
| 16       | 20     | <u>GGAAAGCGGUGCAGGAGCAGG</u> | 21      |
| 37       | 20     | <u>GGAAAGCGGUGCAGGAGCAGG</u> | 21      |
| 58       | 20     | <u>GGAAAGCGGUGCAGGAGCAGG</u> | 21      |
| 79       | 20     | <u>GGAAAGCGGUGCAGGAGCAGG</u> | 21      |
| 100      | 20     | <u>GGAAAGCGGUGCAGGAGCAGG</u> | 21      |
| 121      | 20     | <u>GGAAAGCGGUGCAGGAGCAGG</u> | 21      |

**D**

| Position | Length | QGRS                                  | G-Score |
|----------|--------|---------------------------------------|---------|
| 10       | 29     | <u>GGAAAGCGGUGGAGCAGGAGGUGGUGCUGG</u> | 21      |
| 41       | 23     | <u>GGAGCCUAGGAGGCGGUGCAGGG</u>        | 19      |
| 64       | 29     | <u>GGGAGCAGUGGAGGCAGCGGAGCAGGAGG</u>  | 21      |
| 103      | 26     | <u>GGAGGCAGCGGAGCAGGAGGAGCAGCGG</u>   | 18      |

**Figure 17-** Predicted G4-forming sequences of herpesvirus GMPs repetitive domain.

EBNA1 GAR domain (A), LANA1 QED domain (B), *Papiine herpesvirus* EBNA1ba GAR-like domain (C) and *Macacine herpesvirus* 4EBNA1rh (D) GAR-like GAR domain predicted G4s. By using the software QGRS mapper (<http://bioinformatics.ramapo.edu/QGRS/index.php>) we identified the predicted G4-forming sequences in the 711 bp nucleotidic sequence of the GAR mRNA (according to the B95.8 strain), the 1377 bp nucleotidic sequence of the QED mRNA, the 147 bp corresponding to EBNA1ba and the 141 bp domain corresponding to EBNA1rh. All possible G4 motifs were identified.

## 6. *Concluding remarks*

To conclude, during this thesis we characterized a novel function for the cellular protein NCL on EBV EBNA1-based mechanism of immune evasion which involves the binding of NCL to the GAR domain G4 motifs. We showed for the first time that NCL, besides its already described functions as a 5'- and 3'-UTR RNA-binding protein altering mRNA translation and stability, is also able to alter the translation of some mRNAs by direct binding to their coding regions. Furthermore, we demonstrated that this interaction takes place in the nucleus, due to the normal location of NCL in the cells, and that small G4 ligands are able to disrupt NCL binding from EBNA1 mRNA which enhances EBNA1 expression and antigen presentation, making EBV-infected cells susceptible to T cell recognition and elimination.

As it was mentioned at the beginning of this discussion, at the moment I started my PhD two models were proposed to explain the mechanism involved in GAR-based translation inhibition: the GAR polypeptide and the GAR mRNA structure. Our results proved the influence of GAR mRNA G4s as a recognition signal for NCL binding, but we cannot excluded a possible additional influence of GAR polypeptide in the final mechanism.

In the course of this work, we identified the first cellular factor involved in the GAR-based inhibition of translation effect and a G4 ligand, PhenDC3, which is capable of disrupting the binding of NCL to EBNA1 mRNA, thereby leading to an increase in EBNA1 expression and antigen presentation. Taken together, these results indicate that NCL-GAR mRNA interaction constitutes a new therapeutic target to treat EBV-associated cancers.

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## **Nucleolin is involved in Epstein-Barr virus EBNA1- based mechanism of immune evasion and represents a relevant therapeutic target to treat cancers linked to this virus**

### **ABSTRACT :**

The Epstein-Barr Virus (EBV) is the first oncogenic virus described in humans. It is a ubiquitous virus which infects over 90% of the human population.

EBV causes a latent infection of B lymphocytes and remains asymptomatic in most of infected individuals. However, in some conditions like immunosuppression, EBV can cause uncontrolled cell proliferation responsible for some types of cancers like Burkitt lymphoma, Hodgkin lymphoma and nasopharyngeal carcinoma.

During latency, EBNA1 is the only protein expressed in all EBV-infected cells as it is essential for EBV genome replication and maintenance. EBNA1 is highly antigenic and T cells raised against EBNA1 exist; nevertheless EBV-infected cells are not eliminated by the host immune system. This is due to the central GAR (Gly-Ala repeat) domain of EBNA1 which is able to inhibit the translation of its own mRNA in cis, thereby preventing the recognition of EBV-infected cells by the immune system of the host. The mechanisms involved in this GAR-based translation inhibition were unknown when I started my PhD.

During this thesis, we identified the first cellular factor, Nucleolin (NCL), able to affect GAR-based translation inhibition. We showed that NCL overexpression enhances EBNA1 translation inhibition and inversely, that its downregulation decreases EBNA1 translation inhibition effect. Furthermore, we demonstrated that the interaction between NCL and GAR RNA occurs through NCL binding to GAR-encoding mRNA via a particular secondary structure called G-quadruplex (G4). We showed that disrupting NCL-GAR RNA interaction with small G4 ligands enhances EBNA1 translation and thus antigen presentation, which constitutes a new therapeutic avenue to treat EBV-associated cancers.

Finally, we went deeper into the mechanism involved in this interaction by showing that NCL has a direct effect on the GAR-based translation inhibition and that the nuclear location of NCL-GAR RNA interaction is forced by the nuclear location of NCL in the cells.

Taken together, these results have shed light upon the mechanism involved in the GAR domain translation inhibition and immune evasion, and have revealed a possible therapeutic target to eradicate EBV-associated cancers.